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The kinin receptor is expressed in the Malpighian tubule stellate cells in the mosquito *Aedes aegypti* (L.): a new model needed to explain ion transport?

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Abstract

It is known that insect kinins increase diuresis and fluid secretion in the *Aedes aegypti* Malpighian tubule, causing a rapid drop of the transepithelial resistance and increasing chloride conductance from the hemolymph towards the tubule lumen. The tubule is composed of both principal and stellate cells. The main route for increased chloride influx upon kinin treatment is proposed to be paracellular, with septate junctions acquiring increased chloride selectivity and conductance. Therefore, kinin treatment renders the *Aedes aegypti* tubule a “leaky epithelium”, and under this model the kinin receptor is postulated to be expressed in principal cells. However, in another dipteran, the fruit fly *Drosophila melanogaster*, the main route for chloride transport is transcellular through stellate cells. In both the fruit fly and the mosquito *Anopheles stephensi* the kinin receptor has been immunolocalized in stellate cells, where it regulates transepithelial chloride permeability. Here we show that in *Aedes aegypti*, similarly, the stellate cells express the kinin receptor. This was confirmed through immunohistochemistry with two specific anti-kinin receptor antibodies and confocal analysis. The receptor is detected as a 75kDa band in western blot. These results indicate that the currently accepted model for chloride transport must be re-evaluated in *Aedes aegypti* and suggest the kinin regulatory signals controlling intercellular junctions originate in the stellate cells.

Keywords

insect GPCR (G protein-coupled receptor); *Aedes* kinin receptor; diuresis; leucokinin; Malpighian tubules; stellate cells; chloride transport; neuropeptide

INTRODUCTION

In females of *Aedes aegypti* mosquitoes, a protein rich blood meal is necessary to initiate vitellogenesis for reproduction. When feeding, females typically ingest more than ten times their hemolymph volume in blood. This added weight hinders flight begetting a fitness cost. Thus, females rapidly process the meal and void excess fluid, with the onset of urination observed while still blood feeding. Nearly 40% of the fluid ingested during the meal is excreted during the first hour after feeding (Beyenbach, 2003b). Most of this water load is

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secreted by the Malpighian tubules into the hindgut that excretes it from the body. In adult mosquitoes each Malpighian tubule is a one-cell thick epithelium made up of two types of cells, principal and stellate cells. The principal cells are large and cuboidal with a thick brush border and large nuclei, and the stellate cells are smaller, less abundant, thin, and star-shaped. Septate (tight) junctions lay between these cells (Beyenbach, 2003a). The distal, blind-ended portion of the Malpighian tubules is primarily responsible for ion and water transport from the hemolymph into the tubule lumen for primary urine formation which is nearly isoosmotic to the female hemolymph (Beyenbach et al., 2010). Stellate cells are only present in the distal two-thirds of the tubule (Patrick et al., 2006). The proximal tubule, which opens at the junction of the hindgut pyloric valve and midgut, lacks stellate cells and functions for reabsorption of excess ions and fluid (Beyenbach, 1995). This mechanism drives fluid into the hindgut for further reabsorption and then excretion from the body.

The Malpighian tubules of females of *Ae. aegypti* are not innervated, but are controlled by diuretic hormones in the hemolymph (Coast, 2007). A plethora of neurohormones interact with receptors on the surface of both principal and stellate cells to intricately coordinate ion transport towards the tubule lumen with water following this osmotic gradient. The diuretic and/or antidiuretic hormones produce an intracellular signaling cascade of secondary messengers affecting kinases or other molecules that regulate effectors to move ions across the Malpighian tubule epithelium (for reviews, see (Coast, 2007; Schooley et al., 2005). In the Malpighian tubules of *Ae. aegypti* females there are two routes for ion transport from the hemolymph to the lumen: the transcellular path through either principal or stellate cells, and the paracellular route through septate junctions between cells (Beyenbach, 2003a; Beyenbach, 2003b). The cations sodium and potassium are transported transcellularly through the principal cells (Beyenbach, 2001; Beyenbach and Masia, 2002; Petzel et al., 1999) while the movement of chloride ion may occur through both the paracellular and transcellular routes. The paracellular Cl^- transport through septate junctions between principal cells is supported by electrophysiological studies (Beyenbach, 2003a; Wang et al., 1996). The transcellular Cl^- route through stellate cells is supported by the recent finding of an anion exchanger on their basal membrane (Piermarini et al., 2010) and by the identification of two types of chloride channels in stellate cell apical membrane (O'Connor and Beyenbach, 2001). Chloride transport towards the lumen of the Malpighian tubule of dipterans such as *Drosophila melanogaster* and *Ae. aegypti* is stimulated by the endogenous insect kinins, drosokinin and Aedes-kinins, respectively.

Insect kinins are multifunctional neuropeptide hormones with myotropic and diuretic activity in insects (Nachman et al., 2009). Leucokinin diuretic activity was first discovered in *Ae. aegypti* Malpighian tubules; they depolarize the Malpighian tubule transepithelial voltage by increasing transepithelial Cl^- conductance. The three endogenous *Ae. aegypti* kinins are encoded by a single cDNA; kinins induce hindgut contractions and depolarize the transepithelial voltage of Malpighian tubule increasing fluid secretion (Cady and Hagedorn, 1999a; Veenstra et al., 1997). The Aedes kinins increase intracellular IP_3 in the isolated Malpighian tubule of *Ae. aegypti*, suggesting they act by increasing intracellular calcium levels from IP_3 -sensitive calcium stores (Cady and Hagedorn, 1999b). In agreement with these findings, all three Aedes kinins activate the recombinant *Ae. aegypti* kinin receptor (Protein ID AAT95982.1) expressed stably in CHO-K1 cells, eliciting dose-dependent intracellular calcium release (Pietrantonio et al., 2005b). In the fruit fly, insect kinins increase fluid secretion through Cl^- transport via the stellate cells (O'Donnell et al., 1998; Terhzaz et al., 1999). In *D. melanogaster* and the mosquito *Anopheles stephensi*, the kinin receptors were immunolocalized in stellate cells (Radford et al., 2002b; Radford et al., 2004b). Drosokinin binding to the drosokinin receptor activates Gq resulting in activation of phospholipase C and increase production of IP_3 , causing the release of calcium from

intracellular stores through the IP₃ receptor (Radford et al., 2002b), similar to the above mentioned findings in *Ae. aegypti*.

Despite similarities in insect kinin signaling in the fruit fly and mosquitoes, the cellular localization of the receptor in Malpighian tubules of *Ae. aegypti* remains unresolved. However, Beyenbach's laboratory has published many reports suggesting the presence of the kinin receptor in principal cells of *Ae. aegypti* based on the following findings: 1. Both a calcium ionophore and thapsigargin (a specific inhibitor of calcium uptake by intracellular stores) mimic the effects of leucokinin on principal cells, suggesting roles for extracellular calcium and intracellular calcium release (Yu and Beyenbach, 2002); 2. electrophysiological studies showed that leucokinin could stimulate chloride ion transport in selected regions of the Malpighian tubule devoid of stellate cells, supporting a paracellular route for chloride transport and suggesting stellate cells are not required for leucokinin signaling (Beyenbach, 2003b; Yu and Beyenbach, 2004); 3. *Aedes* kinin-3 causes subunits A and B of the V-ATPase V₁ complex to leave the cytosol of principal cells, consistent with their movement towards the plasma membrane for their assembly to the V₀ complex resulting in a functional V-ATPase (Beyenbach et al., 2009).

Here we show the immunolocalization of *Ae. aegypti* kinin receptor in the stellate cells of the female Malpighian tubules through histochemistry with two different anti-peptide antibodies and that the receptor is expressed as a single form in Malpighian tubules of non-blood fed females.

MATERIALS AND METHODS

Insects

Aedes aegypti (L.) Rockefeller strain (Diptera: Culicidae), were reared at 27 °C as previously described (Pietrantonio et al., 2005b). Larvae were fed high protein Purina ONE® Natural Blends dog food (Nestle Purina PetCare, St. Louis, MO, USA); density was kept low to maximize adult size. Adults were provided 10% sucrose solution to feed *ad libitum*. Females were fed on mice according to NIH guidelines (AUP 2007-141).

Antibodies

Polyclonal anti-peptide antibodies were developed in New Zealand female rabbits by Bethyl Laboratories, Inc. (Montgomery, Texas) and affinity-purified. Antibodies were developed against two regions of the kinin receptor (KR): a sequence towards the N-terminal region (residues 13-32), NNNTLNGSDVEIVKEQDALY, located before the first predicted transmembrane region, and a sequence towards the C-terminus (residues 328-345), immediately outside the predicted seventh transmembrane region, NEKFKREFHKRYPFRGRN (Pietrantonio et al., 2005b). Both peptides were synthesized with an additional N-terminal cysteine for conjugation to keyhole limpet hemocyanin. After purification, the antibodies were kept in Tris-citrate/phosphate buffer pH 7-8 in 0.1% sodium azide, at a concentration of 1.06 mg/ml (anti-KR-Nt₁₃₋₃₂) and of 1.15 mg/ml (anti-KR-Ct₃₂₈₋₃₄₅) and stored at 4°C.

Whole mount immunohistochemistry

The method was followed as previously described with modifications (Duchesne et al., 2003). The digestive systems of non-blood fed and blood fed 3-5-day old female *Ae. aegypti* were dissected in phosphate buffered saline (PBS) and transferred into freshly prepared 4% paraformaldehyde in PBS for fixation. Blood-fed females were dissected 24 h after blood feeding. Tissues were fixed 2 h at room temperature (RT) with slow agitation. The fixatives were removed by 3 × 10 min washes with 70% ethanol, on ice. After rinsed in PBS-0.1%

Tween (PBST) 2 × 5 min at RT, tissues were incubated in 12 µg/ml protease K (Sigma-Aldrich, St Louis, MO, USA) in PBS for 5 min at RT. Tissues were then rinsed in PBST 2 × 5 min at RT and blocked in PBST with 10% normal goat serum (NGS) (Sigma-Aldrich) for 24 h at 4 °C. All subsequent steps were carried out with slow agitation at RT. After blocking, tissues were incubated with primary antibodies diluted in PBSTG (PBST containing 2% NGS) for 24 h. These primary antibodies were: anti-KR-Nt₁₃₋₃₂ diluted 1:40, or anti-KR-Ct₃₂₈₋₃₄₅ diluted 1:10 for *Aedes* kinin receptor localization. Negative controls were with pre-immune serum from rabbit at 1:40 dilution and anti-KR-Nt₁₃₋₃₂ and anti-KR-Ct₃₂₈₋₃₄₅ antibodies pre-absorbed with the respective synthetic N- or C-terminus peptides. Affinity-purified antibodies (~40 mg in 1 ml PBST/2% NGS) were pre-absorbed with 500 µg of antigen-peptide (N or C-terminus peptides, respectively) for 5 h at RT with gentle shaking. Tissues for positive controls were incubated with rabbit anti-aquaporin polyclonal antibody at 1: 250 dilution as previously described (Duchesne et al., 2003) (not shown).

After incubated with primary antibodies, tissues were washed 4 × 20 min in PBSTG. For anti-KR-Nt₁₃₋₃₂ antibodies, tissues were incubated in a 1:200 dilution of biotinylated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA) for 1.5 h. Tissues were washed 4 × 20 min with PBSTG as above and incubated in a 1:200 dilution of Streptavidin-Alexa Fluor 546 (Invitrogen, Carlsbad, CA, USA) for 1 h. For anti-KR-Ct₃₂₈₋₃₄₅ antibodies, tissues were incubated in a 1:200 dilution of Alexa Fluor 546 goat anti-rabbit IgG (Invitrogen) overnight.

Tissues were then washed 6 × 30 min in PBST and mounted on slides with Vectashield™-DAPI (Vector, Burlingame, CA, USA) for nuclear staining. Results were observed under a Carl Zeiss Axioimager A1 microscope with filters for DAPI (G 365 nm, FT 395 nm, BP 445 nm) and Alexa Fluor 546 (BP 546 nm, FT 560 nm, BP 575–640 nm). Images were obtained with an AxioCam MRc color camera (Carl Zeiss) and analyzed with axiovision program (Carl Zeiss). Confocal images were taken of tissues incubated with the anti-KR-Ct₃₂₈₋₃₄₅ antibodies using an Olympus FV1000 confocal microscope at the Microscopy and Imaging Center (TAMU).

Malpighian tubule membrane preparation and western blot

Membrane preparation was as previously described (Lu, et al., 2009). Briefly, Malpighian tubules (M. tubules) were dissected from 3-5 day-old non-blood fed female mosquitoes in PBS and homogenized with a hand-held homogenizer in cold buffer A (25 mM Tris/HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol) containing Complete Protease Inhibitor Cocktail® (ROCHE). The homogenate was centrifuged at 800 g for 5 min and the supernatants were collected and centrifuged at 100,000 g for 1 h at 4 °C. The pellets were re-suspended in cold buffer B (50 mM Tris-HCl, pH 7.5, 2 mM CaCl₂) with protease inhibitors and stored at -80 °C.

For western blot, M. tubule membrane preparations were separated on SDS-PAGE (10%, Bio-Rad, Hercules, CA, USA) and transferred to PVDF (poly-vinylidene difluoride) membranes (Millipore). Membranes were blocked for 1 h at RT in TBST (10 mM Tris base, 140 mM NaCl, 0.1% Tween-20, pH 7.4) containing 5% non-fat milk and then incubated overnight at RT with rabbit anti-KR-Ct₃₂₈₋₃₄₅ antibodies (1:250) in the same solution. Anti-KR-Ct₃₂₈₋₃₄₅ antibodies (1:250 in 5% non-fat milk in TBST) were pre-absorbed with C-terminal peptide antigen (500 µg) overnight at RT and were used as negative controls. After 3 × 10 min washes with TBST, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:40,000) for 1 h. After the same washing steps, the membrane was visualized using the Enhanced Chemiluminescence System (Pierce) on film (Kodak, Rochester, NY, USA).

RESULTS AND DISCUSSION

A missing critical piece of information to contribute to the understanding of kinin function is to identify the location of the kinin receptor in *Ae. aegypti* Malpighian tubules. Here we clearly show the *Ae. aegypti* kinin receptor (GenBank AY596453.1) is immunolocalized in the plasma membrane of stellate cells of Malpighian tubules from both non-blood fed (Fig. 1, A and D, arrows) and blood fed females 24 h after feeding (Fig. 1, B, arrows). Negative controls with preimmune serum (Fig. 1F) or two different affinity-purified antibodies preabsorbed with the respective receptor antigenic peptides (N-terminus, Fig. 1C and C-terminus, Fig. 1E) show no immunoreactivity in the stellate cells (arrowheads). Confocal analysis undoubtedly resolved the kinin receptor expression in the basolateral membrane (hemolymph side) of the stellate cells and not in principal cells (Fig. 2). The high intensity red immunofluorescence signal suggests that the kinin receptor is highly expressed in the stellate cells of *Ae. aegypti*, with similar localization to *D. melanogaster* and *An. stephensi* (Radford et al., 2002b; Radford et al., 2004b).

With respect to the signaling pathway, there is consensus in that the three dipteran recombinant kinin receptors functionally expressed in S2 or mammalian cells increased intracellular calcium in response to insect kinins (Pietrantonio et al., 2005b; Radford et al., 2002b; Radford et al., 2004b). This was also demonstrated for *Drosophila* in intact tissues *in vitro*, including Malpighian tubules (Radford et al., 2002b), and specifically in M. tubule stellate cells and not principal cells (Terhzaz et al., 1999). Contradictory evidence about the location of the receptor had been previously reported for *Ae. aegypti*. Stimulating the *Ae. aegypti* female Malpighian tubules with leucokinin initiates a cascade of intracellular events that ultimately regulate septate (tight) junctions between the principal cells, resulting in a “leaky epithelium” that permits the rapid influx of chloride ions to the tubule lumen (Beyenbach, 2003a; Yu and Beyenbach, 2004). Increases in intracellular calcium in principal cells have also been reported for *Ae. aegypti* tubules stimulated with leucokinins (Yu and Beyenbach, 2002). In this manner, leucokinin is able to increase chloride transport even in regions of tubules apparently devoid of stellate cells, indicating that leucokinin acts through the principal cells, and that stellate cells are not required for kinin signaling (Yu and Beyenbach, 2004). Our results, however, support that stellate cells are the main target of *Aedes* kinin hormones. Perhaps results of Beyenbach’s group showing activity of kinin peptides on principal cells could be explained by kinin activity through a different, yet unidentified receptor in these cells or by movement of secondary messengers from stellate cells towards the principal cells through gap junctions.

Western blot analysis of Malpighian tubules with the anti-KR-Ct₃₂₈₋₃₄₅ antibodies revealed a single band at 75 kDa (Fig. 3). The band is specific because it is not detectable with antigen-preabsorbed antibodies (Fig. 3, lane 2). A faint band at ~40kDa is most likely a degradation product of the receptor. The 75kDa band is slightly higher in molecular weight than the kinin receptor predicted MW of 65.2 kDa. This 10 kDa difference is likely due to post-translational GPCR modifications (Pietrantonio et al., 2005a). In M. tubules of *D. melanogaster* two bands at 60- and 70-kDa are identified in western blots with anti-drosokinin receptor antibodies, similarly developed against a 14-amino acid segment in the receptor C-terminus, and this MW difference is caused by N-glycosylation (Radford et al., 2002a). In *An. stephensi*, also two bands at 65- and 72-kDa are detected in M. tubules with specific antibodies against C-terminal residues 531-543 of the receptor (Radford et al., 2004a). Based on these previous results, Schepel et al. (2010) recently proposed the hypothesis of “glycosylation dependent signaling.” To explain observed differences in the biological activities of different kinin peptide analogs they postulated that two kinin receptor forms, glycosylated and non-glycosylated, may be present in M. tubules of non-blood fed females of *Ae. aegypti*. However, our results show that the kinin receptor encoded by

AY596453.1 is expressed in the M. tubule stellate cell as a single protein form of about 75kDa in non-blood fed females. Therefore, our results are consistent with the Schepel et al. (2010) alternative hypothesis of “ligand-dependent selectivity of receptor signaling, where a single receptor can give rise to more than one physiological response” (Schepel et al., 2010). We can not, however, completely rule out that the *Aedes* kinin receptor may be detectable as an unmodified form (65.2kDa) under specific physiological conditions. Regardless, evidence points to receptor expression solely in the stellate cells.

Additional evidence from previous studies supports that chloride is transported through stellate cells in *Ae. aegypti*, with the kinin receptor regulating chloride movement. First, a recent report on the cloning and immunolocalization of a putative anion exchanger ($\text{Cl}^-/\text{HCO}_3^-$; solute carrier 4 superfamily) on the basal membrane of stellate cells in *Ae. aegypti* is consistent with Cl^- transport through this transcellular route (Piermarini et al., 2010). The functional inhibition of this exchanger with disulfonic stilbene derivatives (DIDS) appears to only negatively affect secretion rates resulting from direct stimulation with diuretic hormone (*Anopheles* calcitonin-like peptide) or *Aedes* kinin-3, but does not significantly inhibit basal rates in isolated tubules (Piermarini et al., 2010). These findings suggest the anion exchanger is a crucial effector only to sustain high rates of diuresis. Second, chloride channels identified in the stellate cells' apical membrane (O'Connor and Beyenbach, 2001) may also provide the exit for chloride that has entered principal cells through the cation-chloride co-transporter via gap junctions (Fig. 4) (Beyenbach et al., 2010). While the molecular identity of these chloride channels is still unknown, one of the *Drosophila* ClC family channel gene (CG6492) might be the candidate ortholog in *Ae. aegypti* because it is overexpressed in Malpighian tubules with respect to the whole fly (Wang et al., 2004). Figure 4 shows a summary of the main hormones, receptors, secondary messengers and transporters involved in fluid secretion in mosquito Malpighian tubules. In addition, based on the microarray expression data from *Drosophila* tubules (Wang et al., 2004), more neuropeptide receptors may be involved in diuresis in the Malpighian tubules of *Ae. aegypti*.

The septate junctions between principal cells are suggested to provide the paracellular route for passive chloride transport upon insect kinin treatment (Beyenbach, 2003a). Gap junctions are also present in *Ae. aegypti* Malpighian tubules and mRNAs for four insect gap junctional proteins: innexins 1, 2, 3 and 7, were detected through RT-PCR (Weng et al., 2008). Intercellular communication through gap junctions was studied after leucokinin application and appears to be relatively insensitive to Ca^{2+} . Gap junctional communication between stellate and principal cells was not particularly addressed in this latter study, and therefore, assumptions of the model presented (Weng et al., 2008) may need to be re-evaluated based on our finding of the kinin receptor in stellate cells. For example, gap junctions may contribute to the transport of signaling molecules such as IP_3 or Ca^{2+} from the stellate to the principal cells upon kinin exposure. This could explain observed changes such as increases in calcium in principal cells upon leucokinin treatment and coupling of the V-ATPase. Therefore, it is necessary to investigate gap junctional communication between stellate and principal cells. In agreement with a previous report (Patrick et al., 2006), our results underscore that the elongated shape of cellular extensions of stellate cells may have confounded Beyenbach's laboratory's findings on the effects of leucokinin in sections of tubule apparently lacking stellate cells (Yu and Beyenbach, 2004) (see long extensions of stellate cells in Fig 1 B and D). Perhaps their assumptions on measurements of gap junctional coupling only between principal cells may have been faulty because long, narrow sections of the stellate cells might have been present (Weng et al., 2008). In *Drosophila*, application of another diuretic peptide, Cap_{2b} , does not induce a response in stellate cells, but only affects principal cells, suggesting that coupling through gap junctions does not occur (Rosay et al., 1997). In contrast, the rapid excretion of an enormous water load in female mosquitoes after a blood meal may have evolved through adaptations such as

regulation of gap junctional communication in addition to the cellular compartmentalization of anion and cation transport and leaky septate junctions (Beyenbach et al., 2010). Our finding that the *Ae. aegypti* kinin receptor is located in stellate cell membranes may require revising the current model for chloride transport across the Malpighian tubule epithelium in this species and indicates that if regulation of intercellular junctions occurs, the signaling pathway for the kinin receptor initiates in the stellate cell.

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Fig. 1.

Whole mount immunohistochemistry of *Aedes aegypti* Malpighian tubule showing the immunolocalization of the kinin receptor in the stellate cells in 3-5 day-old females (red signal). Kinin receptor immunoreactivity (arrows) was detected in the stellate cells of Malpighian tubules from both non-blood fed (A, D and G) and blood fed females (B; 24 h after feeding). A and B: Malpighian tubules were incubated with 1:40 dilution of anti-KR-Nt₁₃₋₃₂ antibodies. D and G: Malpighian tubules were incubated with 1:10 dilution of anti-KR-Ct₃₂₈₋₃₄₅ antibodies. Panel G: complete single M. tubule showing the kinin receptor signal in stellate cells (arrows), which are only present in the distal three fourths of the tubule. The tubule contains ~57 principal cells; the position of a principal cell adjacent to a stellate cell is indicated by numbers at the bottom of the panel beginning at the tip cell. Malpighian tubules as negative controls showed no immunoreactivity (small nuclei in blue, arrowheads, C, E, and F) and were incubated with: C. anti-KR-Nt₁₃₋₃₂ antibodies (1:40 dilution) pre-absorbed with Nt₁₃₋₃₂ peptide antigen; E: anti-KR-Ct₃₂₈₋₃₄₅ antibodies (1:10 dilution) pre-absorbed with Ct₃₂₈₋₃₄₅ peptide antigen; F: pre-immune antiserum (1:40 dilution). Blue nuclei smaller than those of stellate cells correspond to nuclei of tracheolar cells, which are not seen or detected above background (*i.e.* nuclei in 1.C, right corner).

Fig. 2.

Confocal microscopy image of a female *M. tubule* showing kinin receptor immunofluorescence. A. Longitudinal view (40 images stacked, each obtained every 1.5 μm ; 61.72 μm of tissue analyzed) of the distal tubule showing receptor immunostaining only in stellate cells; two stellate cell nuclei are indicated with arrows. B. Single confocal optical section showing the basolateral membrane receptor immunostaining in the stellate cell from panel A (top arrow). The stellate cell apical membrane, towards the tubule lumen, is not labeled and cannot be visualized here (dashed line). Notice that the receptor signal stops at the edge of the stellate cell (arrow). The adjacent principal cell membrane is not immunolabeled (arrowheads). C. Cross sectional view of the same stellate cell in B showing receptor signal only in the basolateral membrane.

Fig. 3. Western blot analysis of kinin receptor expression in membranes from Malpighian tubules of females of *A. aegypti* (100 μ g of protein per lane). The blot was incubated with a 1:250 dilution of anti-KR-Ct₃₂₈₋₃₄₅ antibodies (lane 1), and with a 1:250 dilution of antigen-preabsorbed anti-KR-Ct₃₂₈₋₃₄₅ antibodies as a negative control (lane 2). Antibodies detect only one band at 75 kDa in lane 1. No bands are detected in the negative control, as expected.

Fig. 4.

Primary ion transport systems and receptor signaling pathways in Malpighian tubules of female mosquitoes. In principal cells, a vacuolar-type H⁺ ATPase (V-ATPase) energizes the apical membrane by transporting protons to the lumen. The putative cation/proton antiporter that transports protons back to the cytosol has not been identified in *Ae. aegypti*; however, the sodium/proton antiporter (NHA1) has been immunolocalized in apical membranes in larvae of *An. gambiae* (Rheault et al., 2007). CPA2 proteins functionally similar to the alkali-metal/proton antiporter (Wieczorek exchanger) are expressed in apical membranes of *Drosophila* M. tubules (Day et al., 2008). In *Ae. aegypti* females, a cation/proton exchanger (NHE8) was immunolocalized in principal cells by two different laboratories and may be located both in the luminal plasma membrane and subapically. Cations move from the hemolymph through the sodium/proton (Na⁺/H⁺) exchanger (NHE3), the cation-chloride (Na⁺/K⁺/Cl⁻) co-transporter, and a potassium channel (For reviews, see Beyenbach et al., 2010; Schooley et al., 2005). The P-type Na⁺/K⁺ ATPase is present in principal cells in the proximal tubule (not shown) (Patrick et al., 2006). In stellate cells, a P-type Na⁺/K⁺ ATPase is putatively present in the basolateral membrane (Patrick et al., 2006) and an anion (Cl⁻/COH₃⁻) exchanger transports Cl⁻ into these cells (Piermarini et al., 2010). Chloride reaches the tubule lumen via chloride channels in stellate cells (O'Connor and Beyenbach, 2001; O'Donnell et al., 1998) and through septate junctions. Gap junctions between cells may provide a spectrum of perm-selectivity from ions to secondary messengers and increase the potential for regulating communication through gap junctions (Weng et al., 2008). Hormones bind receptors on the basolateral membranes to elicit their respective effects: (1) (Leuco)kinin receptors signal via Ca²⁺ and regulate chloride transport from stellate cells (this paper; Pietrantonio et al., 2005b; Veenstra et al., 1997; Yu and Beyenbach, 2002; Yu and Beyenbach, 2004); (2) Corticotropin releasing factor (CRF)-like diuretic hormone 44 (DH₄₄) receptors signal via cAMP, regulating cation transport (Coast et al., 2005); (3) Calcitonin-like diuretic hormone 31 (DH₃₁) receptors signal via cAMP to regulate sodium transport (natriuresis) (Coast et al., 2005; Jagge and Pietrantonio, 2008); and (4) Periviscerokinin (CAP_{2b} or CAPA) receptors signal through cGMP and reduce secretion in *Aedes* females (Schooley et al., 2005).