Short Communication

Na\(^+\)/K\(^+\)-ATPase Activation by cAMP in the Midgut of Lutzomyia longipalpis (Lutz & Neiva, 1912; Diptera: Psychodidae)

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Received 9 November 2021; Editorial decision 18 January 2022

Abstract

Lutzomyia longipalpis (Lutz & Neiva, 1912) females have been intensively studied regarding the regulation of midgut pH. The mechanisms involved in pH regulation are complex, and some aspects remain to be clarified. Here, we investigated the role of the Na\(^+\)/K\(^+\)-ATPase pump as an electrochemical potential generator and its modulation by the second messenger cAMP in the midgut of female L. longipalpis. Our results suggest that not only may Na\(^+\)/K\(^+\)-ATPase be the main generator of an electrochemical potential across membranes in the midgut of female L. longipalpis, but also its activity is positively regulated by cAMP. cAMP-mediated Na\(^+\)/K\(^+\)-ATPase pump activity might be necessary to maintain the transport of the nutrients produced during blood digestion.

Key words: sandfly, Na\(^+\)/K\(^+\)-ATPase, cAMP, midgut, pH
events during blood digestion, such as the stimulation of the Na+/K+-ATPase. Given the high transport demand during digestion, an extra activity to maintain the membrane potential under normal conditions could be useful.

Thus, this study aimed to investigate the role of Na+/K+-ATPase as an electrochemical potential generator and its modulation by the second messenger cAMP in adult L. longipalpis' midgut.

Materials and Methods

Insects

Sand flies were maintained according to Modi and Tesh (1983). Experiments were conducted with 3- to 6-d-old female L. longipalpis.

Measurement of Na+/K'-ATPase Activity

The Na+/K'-ATPase assay was adapted from MacVicker et al. (1993) and Nepomuceno et al. (2017). The midguts of 36 females were dissected and transferred to 360 μl of 50 mM Tris pH 7.3. The sample was sonicated for 20 s and centrifuged at 3,000 g for 3 min. The sediment was resuspended in 360 μl of 50 mM Tris pH 7.3 containing 0.1% Tween-20 and maintained on ice until use.

Typically, six different experimental groups were evaluated: sample plus Mg2+, sample plus Mg2+/Na+, sample plus Mg2+/K+, sample plus Mg2+/Na+ + K+, sample plus Mg2+/Na+ + K+ + ouabain (a Na+/K'-ATPase inhibitor), and boiled sample (ATPase inactivation by preheating at 100°C for 2 min) plus Mg2+/Na+ + K+.

The reaction tube containing Mg2+/Na+ + K+ was prepared as follows: 30 μl of the sample (equivalent to three midguts), 25 μl of 12 mM MgCl2 dissolved in 200 mM Tris pH 7.3; 10 μl of 1 mM NaCl; 10 μl of 150 mM KCl; 2 μl of 100 mM EGTA, 5 μl of 60 mM ATP. When required, distilled water was added to complete 100 μl. The final concentrations were as follows: 3 mM Mg2+, 100 mM NaCl, 15 mM KCl, 2 mM EGTA, 65 mM Tris/HCl pH 7.3, 3 mM ATP. The preparation was incubated at 30°C for 60 min. The ATP stock solution had its pH adjusted to 6.3 with sodium bicarbonate. When required, 10 μl of 10 mM ouabain (Fluka-75640, final concentration 1 mM) was added to the reaction medium. After incubation, 70 μl of distilled water was added, and the amount of inorganic phosphate released was measured with a commercial phosphate dosage kit (Labtest Diagnóstica, Brazil), as described by Nepomuceno et al. (2017).

The other reactions were prepared by adding or omitting some of the ingredients mentioned above. The assays were repeated six times, with two biological replicates by assay.

Na+/K'-ATPase Activation by cAMP or Forskolin

In the present study, we investigated if dibutyryl-cAMP (Sigma D0627), a membrane-permeable cAMP analog, or forskolin (Sigma F3917), an adenylyl cyclase activator, could activate the Na+/K'-ATPase. The protocol was adapted from Nepomuceno et al. (2017).

Twenty-two midguts were dissected in Ringer solution (155 mM NaCl; 10 mM KCl; 2 mM CaCl2; 2 mM MgCl2; 2.7 mM sodium glutamate; 2.7 mM malic acid; 10 mM glucose; 10 mM Tris—pH 7.2) and separated into two tubes (11 midguts for each tube), each containing 100 μl of Ringer solution. The tubes were centrifuged at 3,000g for 3 min, and the supernatant was discarded. The midguts from the experimental groups were preincubated, for 6 min, with 10 μl of Ringer solution containing 2.8 mM db-cAMP, or with 100 μM forskolin. The control group was preincubated only with Ringer solution.

After the preincubation, 330 μl of homogenization solution (0.3 mM sucrose; 0.1 mM EGTA; 0.1 mM imidazole, pH 7.2; 0.1% β-mercaptoethanol; 1 μg/ml pepstatin A; 1 μg/ml E64; 1 mM okadaic acid) were added to each tube, and the midguts were sonicated for 20 s. Pepstatin and E64 are protease inhibitors; okadaic acid is a phosphatase inhibitor.

A typical assay was prepared as follows: to 30 μl of sample (equivalent to three midguts) were added 10 μl of 1 M NaCl; 10 μl of 300 mM KCl; 10 μl of 50 mM MgCl2; 5 μl of 10 mM EGTA; 5 μl of 200 mM imidazole pH 7.2; 5 μl of 600 mM sucrose; 5 μl of 0.2% β-mercaptoethanol; 5 μl of 60 mM ATP. Distilled water was added to complete 100 μl. The final concentrations were, respectively: 100 mM NaCl; 30 mM KCl; 5 mM Mg2+; 0.5 mM EGTA; 100 mM imidazole pH 7.2; 30 mM sucrose; 0.01% β-mercaptoethanol; 5 mM ATP. The ATP stock solution had its pH adjusted to 6.3 with sodium bicarbonate. When required, it was added to the reaction medium, 1 mM ouabain, or 2.8 mM db-cAMP, or 100 μM forskolin (final concentrations).

In summary, the tests were performed as follow: total ATPase activity (assays without db-cAMP, forskolin or ouabain), total ATPases minus Na+/K'-ATPase (assays with ouabain), db-cAMP-stimulated Na+/K'-ATPase (assays with db-cAMP), forskolin-stimulated Na+/K'-ATPase (assays with forskolin), db-cAMP-stimulated Na+/K'-ATPase plus ouabain (assays with db-cAMP and ouabain) and forskolin-stimulated Na+/K'-ATPase plus ouabain (assays with forskolin and ouabain).

The phosphate dosage was performed as described above. Each assay was performed in quintuplicate, and the experiments were repeated five times.

Statistical Analysis

Statistical analysis was performed using Graph Pad Prism 7.0. Data normality was assessed by the Kolmogorov–Smirnov test. The data were analyzed by ANOVA, followed by Tukey’s multiple comparison test, and the results were considered statistically significant when P < 0.05.

Results

The Main Electrochemical Potential Generator in the Midgut Appears to be Na+/K'-ATPase

The experiments were performed to measure the total ATPase activity in the female midgut. The results indicate Na+/K'-ATPase pump would be responsible for the generation and maintenance of the transmembrane potential in the enterocytes of adult insects.

As shown in Fig. 1, total enzymatic activity is reduced from 1.55 ± 0.29 μmol PO4−3 h−1 in the control group (incubated with Mg2+/Na+/K+) to 0.36 ± 0.03 μmol PO4−3 h−1 when ouabain, a specific Na+/K'-ATPase inhibitor, was present in the reaction medium (Mg2+/Na+/K+ + ouabain).

CAMP Stimulates Na+/K'-ATPase Activity in the Midgut

When midguts were pretreated with dibutyryl-cAMP (db-cAMP), it was observed a significant increase in total ATPase activity, from 1.16 ± 0.21 μmol PO4−3 h−1 (control) to 1.5 ± 0.21 μmol PO4−3 h−1 (P < 0.0001; Fig. 2). However, the ATPase activity was greatly reduced when Na+/K'-ATPase was inhibited by ouabain, even in midguts prestimulated by cAMP.
An increase in activity was also observed when cAMP production was stimulated by forskolin (Fig. 3). The total ATPase activity increased from 1.40 ± 0.12 μmol PO\textsubscript{4}^-\textsubscript{3} h\(^{-1}\), in the control group, to 1.70 ± 0.14 μmol PO\textsubscript{4}^-\textsubscript{3} h\(^{-1}\) (P < 0.0001) in the pretreated midguts. As expected, the presence of ouabain inhibited the stimulating effects promoted by forskolin.

Discussion

The decrease in total ATPase activity in the presence of ouabain (Fig. 1) suggests that the Na\(^+\)/K\(^+\)-ATPase is responsible for approximately 75% of total ATPase activity in *L. longipalpis*. Previous studies have shown that in the midgut of female *Anopheles stephensi*, Na\(^+\)/K\(^+\)-ATPase activity responds to at least 80% of total ATPase activity (MacVicker et al. 1993), while in the midgut of female *A. aegypti*, it responds to approximately 70% of this activity (Nepomuceno et al. 2017). Given that the apical V-ATPases are inactive during blood digestion to preserve the midgut alkalinity, the pump responsible for maintaining the membrane potential in the midgut is most likely the Na\(^+\)/K\(^+\)-ATPase. Unlike in adult sandflies and mosquitoes, in larvae the V-ATPase is basolateral and pumps H\(^+\) from enterocytes to the hemolymph in specific areas of the midgut (Onken and Moffet 2009). In this case, it contributes to the generation of the electrochemical potential, as its position on the enterocytes does not compromise the midgut alkalinity characteristic of the larval midgut.

During blood digestion, the electrochemical potential generated by Na\(^+\)/K\(^+\)-ATPase is especially important for the transport of amino acids; either by Na\(^+\)-dependent transporters, enabling the insect to manage the high levels of Na\(^+\) present in the ingested blood (Gill et al. 1998, Boudko 2012, Pacey and O’Donnell 2014), or by H\(^+\)-dependent transporters (Boll et al. 2004, Evans et al. 2009, Nepomuceno et al. 2020). The symport of H’/amino acids helps to remove H\(^+\) ions from the intestinal lumen, which contributes to the alkalization necessary for blood digestion in *L. longipalpis*. Possibly, this symport system has the same physiological role in other hematophagous Diptera from the suborder Nematocera (Nepomuceno et al. 2020).

In *L. longipalpis*, cAMP participates in the abdominal midgut alkalization by activating a bicarbonate/chloride antiport system responsible for pumping bicarbonate ions into the gut lumen in exchange for chloride (Nepomuceno et al. 2021). In fact, second messengers are small molecules that reach intracellular targets, promoting different cellular effects (Lewis et al. 1990), and here we provide evidence that cAMP can positively modulate Na\(^+\)/K\(^+\)-ATPase activity. Pacey and O’Donnell (2014) measured the transepithelial potential before and during blood digestion in *A. aegypti*; according to their results, the membrane potential increased and remained above its basal level during all digestion.

Na\(^+\)/K\(^+\)-ATPase activation by cAMP has also been observed in mammals, where it is associated with the cAMP–protein kinase A pathway (Kirotycheva et al. 1999). A similar activation pattern may occur with the Na\(^+\)/K\(^+\)-ATPase located in insects’ midguts.

In the present study, we provide initial evidence that Na\(^+\)/K\(^+\)-ATPase may be the principal generator of electrochemical potential across the enterocyte’s membranes of female *L. longipalpis* and that this pump activity could be positively modulated by the
second messenger cAMP. The increase in the pump activity stimulated by cAMP could support the transport of nutrients during blood digestion.

Acknowledgments

This work was supported by Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Instituto Nacional de Ciência e Tecnologia—Entomologia Molecular (INCT-FEM), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). We are grateful to Cesar Nonato de Oliveira for his technical assistance with the phlebotomine sandfly colony.

Author Contributions

D.B.N.: conceptualization, formal analysis, investigation, methodology, writing-original draft; G.C.D.P.: funding acquisition, resources, writing-original draft; R.N.A.: funding acquisition, resources, writing-original draft; L.B.K.: funding acquisition, resources, writing-original draft; M.R.V. S.: funding acquisition, resources, writing-original draft; M.H.P.: funding acquisition, resources, supervision, writing-original draft; N.F.G.: conceptualization, data curation, funding acquisition, investigation, methodology, resources, supervision, writing-original draft; R.N.A.: funding acquisition, resources, writing-original draft; M.H.P.: funding acquisition, resources, writing-original draft; R.N.A.: funding acquisition, resources, writing-original draft; N.F.G.: conceptualization, data curation, funding acquisition, investigation, methodology, resources, supervision, writing-original draft; R.N.A.: funding acquisition, resources, writing-original draft; R.N.A.: funding acquisition, resources, writing-original draft; N.F.G.: conceptualization, data curation, funding acquisition, investigation, methodology, resources, supervision, writing-review and editing.

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