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Ultrastructure and Osmoregulatory Function of The Branchial Chamber in the Larvae of Dragonfly, *Libellula lydia* (Odonata)

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ABSTRACT

The ultrastructure of the cells, Na^+, K^+-ATPase activity and immunolocalization were examined in the barnchial chamber of *Libellula lydia* (Drury, 1773) larvae. Na^+,K^+-ATPase activity and localization were performed through biochemical techniques and immunofluorescence light microscopy using a mouse monoclonal antibody IgG α_5, respectively. The branchial chamber possesses six pair gills lamellae that extend into the rectal lumen. A thickened epithelial layer and a modified fat body cells layer are present at the base of the each gill lamella. Epithelial cells covered by a thin cuticle and they possess apical microvilli and baso-lateral membrane infoldings associated with mitochondria. The cytoplasm of the modified fat body cells is filled with mitochondria, glycogen and a few lipid droplets. The Na^+,K^+-ATPase activity was significantly higher (15.36 µM Pi mg^{-1} protein h^{-1}) in the branchial chamber. Na^+,K^+-ATPase immunofluorescence staining was observed in the epithelial layer cells of the basal pads of the rectal gill lamellae, with a consistently high immunoreactivity. These findings show that the epithelial cells present cytological features of the ionocytes, a high activity and concentration of Na^+,K^+-ATPase, confirming their participation in osmoregulation through active ion exchanges.

Keywords: Anisoptera, Branchial chamber, Dragonfly, Immunolocalization, Larvae, Na^+,K^+-ATPase

INTRODUCTION

Anisoptera (Drangonflies) are an interesting group of insects because their larvae are fully aquatic while the adults are terrestrial. The insect's larvae, living in freshwater, are generally hyper-osmotic to their environment. Under these conditions, water tends to pass into the insects, and they are faced with a continuous osmotic inflow of water and with a continuous loss of ions. Adaptation to this environment is primarily achieved by the process of osmoregulation. In insects, the major role in maintaining ionic and water balance seems to be played by the concerted action of the gut and the tissue connected to it, such as the mapighian tubules (Leader and Green, 1978; Nicholls, 1983; Zeiske, 1992). In general, the gut of insects is composed of three major regions: foregut, midgut and hindgut. The hindgut is divided into a thin ileum and a rectum (Chapman, 1998). The rectum of the Odonata larvae have been the subject of qualitative anatomical, physiological, histological, and ultrastructural investigations (Greven and Rudolph, 1973; Wichard and Komnick, 1974; Komnick, 1978; Green, 1979; Komnick and Achenbach, 1979; Miller, 1994; Kohnert *et al*., 2004). In dragonfly larvae, the rectum is divided into a large anterior branchial chamber, housing the heavily tracheated respiratory gill lamellae, and a short

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posterior vestibule (Green, 1979; Kohnert *et al*., 2004).

Na^+,K^+-ATPase (sodium-potasium adenosinetriphosphatase) is the plasma-membrane-associated enzyme which catalyses ubiquitous ATP-driven Na^+/K^+ transport. This enzyme is crucial to ion and water regulation in the fish kidney (Venturini *et al*., 1992; Nebel *et al*., 2005), the gills and antennal glands of crustaceans (Lignot *et al*., 2005; Khodabandeh *et al*., 2005a, b and c) and the Malpighian tubules of insects (Zeiske, 1992; MacVicker *et al*., 1993; Emery *et al*., 1998). Na^+,K^+-ATPase function and activity has been characterized in some insects and has been shown to be essential to excretory and osmoregulatory functions (Anstee and Bell, 1975; Farmer *et al*., 1981; Peacock, 1981; Nicolson, 1993; Emery *et al*., 1998). Recently, immunohistochemical localization of Na^+,K^+-ATPase has been recognized as a useful method for locating the ionocytes in tissues and organs of the crustacean during embryonic and post-embeyonic development (Cieluch *et al*., 2004; Khodabandeh *et al*., 2005a, c; Lignot *et al*., 2005).

Although a good amount of information is now available on the Na^+,K^+-ATPase activity and ion regulatory capacity of the osmoregulatory organs in terrestrial insects (Maddrell, 1977; Green, 1979; Emery *et al*., 1998), the number of investigations on the Odonata is limited. Odonata larvae live in freshwater and take a long time to mature (several years) and they undergo various molts before metamorphosing into adults. They are thus an interesting model for osmoregulatory investigations. No study has been conducted on the localization and activity of Na^+,K^+-ATPase in Odonata larvae except for the pioneering observations of Komnick and Achenbash (1979) on *Aeshna* larvae. The aim of this study was to describe the ultrastructure of cells, Na^+,K^+-ATPase activity and immunolocalization in the branchial chamber of an Odonata larvae, *Libellula lydia*.

MATERIALS AND METHODS

Electron Microscopy

L. lydia nymphs were caught in rivers of Urmieh (West Azarbaijan) in Spring 2003. Six to ten individuals were observed for each experiment. For scanning electron microscopy (SEM), samples were placed in cold 4% glutaraldehyde in 0.1 M phosphate buffer, pH=7.4, containing 5% sucrose. After an initial 1 hour fixation, followed by rinsing in 0.1 M phosphate buffer containing 5% sucrose, the samples were postfixed for 1 hour in 2% osmium tetroxide in 0.1 M phosphate buffer, pH=7.4, containing 5 % sucrose.

For transmission electron microscopy (TEM), the branchial chambers of the samples were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 24 hours at room temperature, pH=7.4. They were then rinsed in sodium cacodylate buffer, and post-fixed for 1 hour in a mixture (V/V) of 2% osmium tetroxide and 0.45 M sodium cacodylate buffer at room temperature. Samples were washed in distilled water and dehydrated in a graded ethanol series and propylene oxide, then embedded in Spurr's resin. Semithin and ultrathin sections were cut on a Reichert OMU3 ultramicrotome. The first sections were stained with toluidine blue. Ultrathin sections were contrasted with 2% uranyl acetate in 70% alcohol and lead citrate, and they were observed on a JEOL 1200 EX2 transmission electron microscope at 70kV (Khodabandeh *et al*., 2005b).

Na^+,K^+-ATPase Activity

The Na^+,K^+-ATPase activities in the branchial chamber of the dragonfly larvae were determined according to the technique described by Norby (1988). The samples were quickly excised, weighed and homogenized in a cold imidazole buffer (0.2 gr/4ml):(50 mmol/L imidazole, 250 mmol/L

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sucrose, and 5 mmol/L EDTA at pH=7.4 with HCl). The cuvette contained 2 ml of reaction mixture with and without 5 mmol/L ouabain (a specific inhibitor of the Na^+,K^+-ATPase). The composition of the reaction media was: 25 mmol/L Tris-HCl; 2 mmol/L MgCl2; 0.25 mmol/L EGTA at pH=7.4; 100 mmol/L NaCl, 25 mmol/L KCl; 1.5 mmol/L PEP; 0.15 mmol/L NADH; 5 mmol/L ATP; LDH/PK enzymes. Incubation was conducted at 37°C for 30 minutes and the Na^+,K^+-ATPase reaction was initiated by the homogenate addition. The Na^+,K^+-ATPase activity was expressed as the activity in the presence of ouabain subtracted from the activity obtained in the absence of ouabain. Results are expressed in terms of both mg tissue (fresh weight or wet weight) and mg protein. Protein concentrations were determined according to the modified procedure described by Lowry *et al.* (1951).

Na^+,K^+-ATPase Immunolocalization

Immunolocalization of the Na^+,K^+-ATPase was performed through immunofluorescence light microscopy using a mouse monoclonal antibody $IgG\alpha_5$ raised against the α-subunit of the chicken Na^+,K^+-ATPase (Takeyasu *et al.*, 1988). In crustaceans, this antiserum has previously been used in the study of the branchial cavity (Lignot *et al.*, 2001), antennal glands and intestine (Khodabandeh *et al.*, 2005b, c) of *Homarus gammarus* (Linnaeus, 1758), the gills (Lignot *et al.*, 2005), and antennal glands (Khodabandeh *et al.*, 2005a) in *Astacus leptodactylus* (Eschscholtz, 1823). Following 24 hours in Bouin's fixator and embedment in paraplast, 5 µm sections were cut on a Leitz Wetzlar microtome and collected on poly-L-lysine-coated slides. The sections were preincubated for 10 minutes in 0.01 mM Tween 20, 150 mM NaCI in 10 mM phosphate buffer, pH=7.3, and then treated with 50 mM NH4Cl in phosphate-buffered saline (PBS), pH=7.3, for 5 minutes to mask the free aldehyde groups of the fixative. The sections were washed in PBS and incubated for 10 minutes with a blocking solution (BS) containing 1% bovine serum albumin (BSA) and 0.1% gelatin in PBS. The primary antibody diluted in PBS to 20 µg/ml was placed on the sections and incubated for two hours at room temperature in a moist chamber. The sections were then incubated for one hour in the secondary antibody (fluorescein isothiocyanate conjugated, FITC) under dark conditions. The slides were rinsed in BS, and were mounted in a medium for fluorescent microscopy (Sigma, ref. 7534) to retard photobleaching. Negative control sections were incubated in BSA-PBS without a primary antibody. The sections were examined under a fluorescent microscope (Leitz Diaplan coupled to a Ploemopak 1-Lambda lamp) with the appropriate filter set (filters of 450 nm to 490 nm).

Statistical Treatment of the Data

Na^+,K^+-ATPase activity data are presented as Means ± SD. The paired Student's *t*-test was used for the significant difference ($P<0.05$) as the confidence limit.

RESULTS

General Organization and Ultrastructure

The rectum is divided into an anterior branchial chamber and a posterior vestibule (Figure 1B). The rectal branchial chamber is richly tracheated by dorsal and ventral tracheal trunks (Figure 1B). It consists of longitudinally oriented curtain-like folds that extend into the rectal lumen from six pairs gill lamellae (Figures 1C and 2B). In cross-section, a thickened basal epithelial layer and a modified fat body cell layer are present at the base of the each gill lamellae (Figures 1D and 2B-E). Ultrastructuraly, the basal epithelial cells of the gill lamellae present extensive basal membrane infoldings associates with more numerous mitochondria and apical microvilli which simultane-

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ously provide extracellular channels (Figures 1F-H). The modified fat body cells are closely applied to the basal membrane (Figure 1E). They possess several different morphologies from fat body cells, such as mitochondria, glycogen, a few lipid droplets and vacuoles (Figure 1E).

Na^+, K^+-ATPase Activity

In the branchial chamber the mean Na^+, K^+-ATPase activity (n=10) was 15.36 µM Pi mg^{-1} protein h^{-1} and 0.78 unit per g of tissue.

Immunolocalization of Na^+, K^+-ATPase

Immunofluorescence microscopy showed consistent results within different cells of the branchial chamber. The positive control lobster branchial cavity was constantly brightly immunostained (Figure 2A). A positive and strong fluorescence of Na^+,K^+-ATPase was found in basolateral sides of the basal epithelial cells of the gill lamellae (Figures 2B-E). No immunofluorescence was detected in the gill lamellae and in the basal fat body cells (Figures 2C-E). Auto-fluorescence was observed on the cuticle (Figure 2B).

DISCUSSION

Ultrastructuraly, the basal epithelial cells of the gill lamellae in *L. lydia* larvae, present ion transporting epithelia characters (chloride cells), such as extensive basal membrane infoldings associates with more numerous mitochondria and apical microvilli. In many orders of insects that possess freshwater larvae these chloride cells are on the general external body surface. In these insects, specialized regions of the external body surface are modified to ion transporting epithelia and take up ions from the medium (Komnick, 1978). They have an abdominal location in Trichoptera and an anal location in Diptera (Komnick, 1978; Komnick and Ashenbash, 1979).

Na^+,K^+-ATPase provides at least part of the driving force for the transepithelial movement of monovalent ions across transporting tissues in many aquatic animals including the invertebrates. Previous observations led to the suggestion that the maintenance of osmotic and ionic balance is directly related to the presence and activity of Na^+,K^+-ATPase in these tissues of insects (Peacock, 1981; MacVicker *et al.*, 1993; Linton and O'Donnell, 1999; Caruso-Neves and Lopes, 2000; Gatto *et al.*, 2000) and crustaceans (Péqueux, 1995; Lucu and Towle, 2003; Khodabandeh *et al.*, 2005a, b and c). The present study is the first to have demonstrated Na^+,K^+-ATPase activity, and the use of a mouse monoclonal antibody IgGα_5 (raised against the α-subunit of the chicken Na^+,K^+-ATPase) for immunoloc-alization of this enzyme, is the first record in the insects, osmoregulatory tissues. These results revealed that the Na^+,K^+-ATPase activity is significantly higher in the branchial chamber of Anisoptera *L. lydia*. In immunocytochemical study, the mouse monoclonal IgGα_5 appears to recognize its epitope in the basal epithelium cells of the rectal gill lamellae. In Na^+,K^+-ATPase immunolocalization, the high antibody specificity may be attributable to the conservation of the α-subunit of the protein in the animal kingdom during the course of evolution, as illustrated in *Artemia franciscana* (Kellogg, 1906; Macias *et al.*, 1991). From the combination of observations conducted by electron microscopy, Na^+,K^+-ATPase activity and immunolocalization, we conclude that the basal epithelial of the gill lamellae in *Libellula* larvae lined by ionocytes (chloride cells) which are the site of ion pumping. They are known to have a similar function to the anal papillae of mosquitoes which are the site of uptake of sodium and chloride ions from the medium (reviewed by Stobbart and Shaw, 1974). The presence of the high levels of Na^+,K^+-ATPase activity and strong immunoreactivity suggest a force activity in

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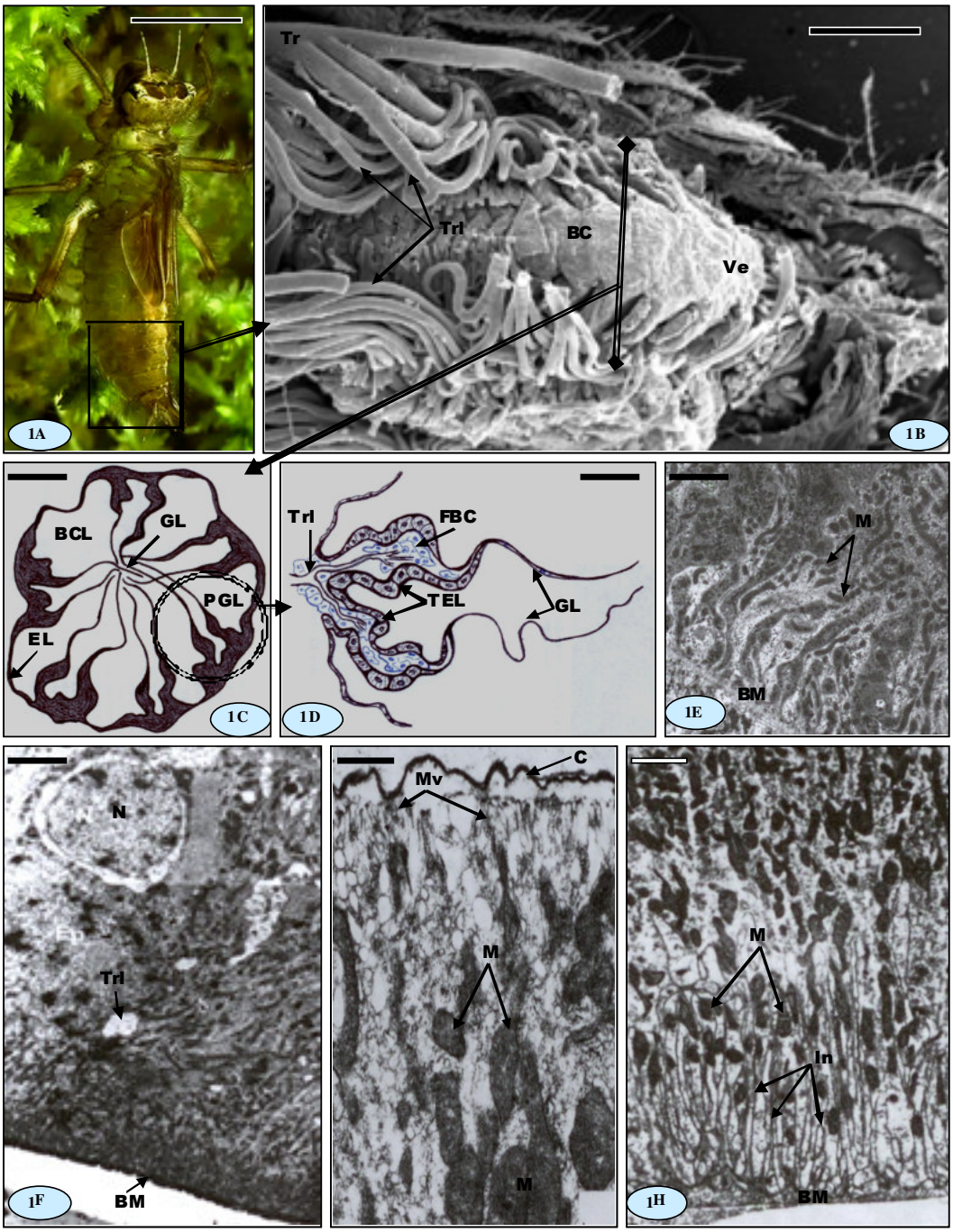

Figure 1. General view, scanning (B) and transmission (E-H) electron microscopy micrographs of the branchial chamber in the dragonfly *L. lydia* larvae. (A). Larvae, (B). Internal general view, (C). Schematic cross-section, (D). Schematic section of a pairs gill lamellae, (E). Basal fat body cell, (F-H). Basal thickened epithelial layer cell, (G). Apical part, (H). Basal part. Abbreviations: BC= Branchial chamber; BCL= Branchial chamber lumen; BM= Basal membrane; C= Cuticle; EC= Extracellular channels; EL= Epithelial layer; FBC= Fat body cells; GL= Gill lamellae; HS= Haemolymph space; In= Infoldings; M= Mitochondria; Mv= Microvili; N= Nucleus; PGL= Pair gill lamellae; TEL= Thickened epithelial layer; Tr= Trachea; TrT= Tracheal trunk; Ve= Vestibule. Bars: 1 cm (1A), 400 µm (1B), 150 µm (1C), 60 µm (1D), 2 µm (1E), 2 µm (1F), 500 nm (1G), 250 nm (1H).

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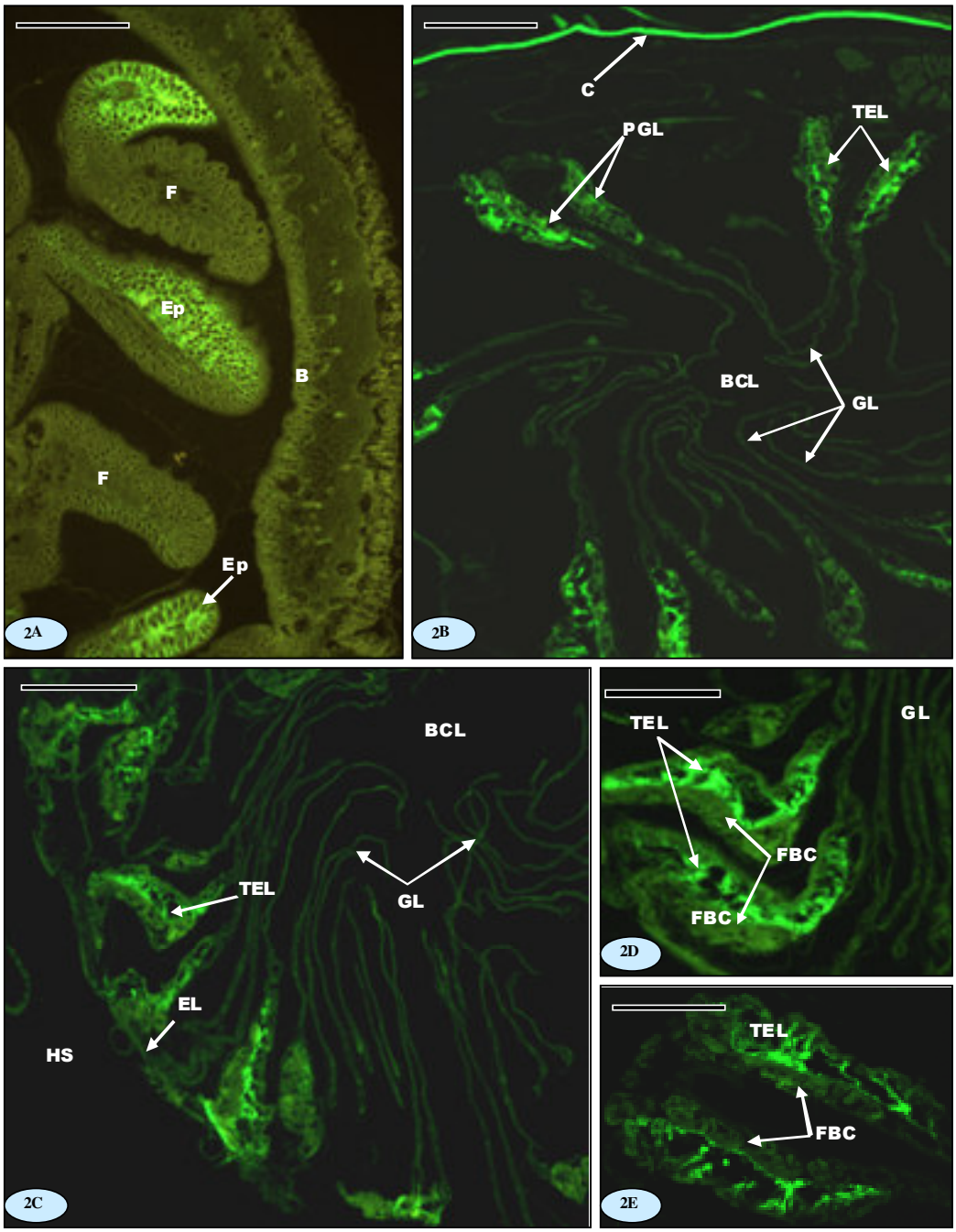

Figure 2. Immunolocalization of Na^+,K^+-ATPase in the branchial chamber of dragonfly *L. lydia* larvae. (A). Positive control from lobster branchial cavity, (B, C). Cross-section of the branchial chamber, (D). A pairs gill lamellae, (E) Basal thickened epithelial layer. Free arrows indicate the immunofluorescent activity of Na^+,K^+-ATPase. Abbreviations: B= Branchiostegite; BCL= Branchial chamber lumen; C= Cuticle; EL= Epithelial layer; Ep= Epipodite; FBC= Fat body cells; GF= Gill filament; GL= Gill lamellae; HS= Haemolymph space; PGL= Pair gill lamellae; TEL= Thickened epithelial layer. Bars: 125 µm (2A), 120 µm (2B), 100 µm (2C), 50 µm (2D, 2E).

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these cells. It permits larvae to cope with the low osmolality of freshwater through hyper-osmoregulation. This result is in agreement with results of Phillips and Audsley (1995) that have shown Na^+,K^+-ATPase is abundant in the lateral membranes of rectal pads in hindgut of the locust, *Schistocerca gregaria* (Forskal, 1775). The biochemical chloride localization indicates that these cells are also able to accumulate chloride ions at the lumen side (Wichard and Komnick, 1974; Komnick and Achenbach, 1979). This property was also found in fish (Venturini *et al*., 1992; Varsamos *et al*., 2002; Nebel *et al*., 2005) and crustacean ionocytes (Lucu and Towle, 2003; Cieluch *et al*., 2004; Khodabandeh *et al*., 2005a, b and c; Lignot *et al*., 2005). No trace of Na^+,K^+-ATPase was detected in the basal fat body cells and in the gill lamellae of *L. lydia* branchial chamber, and they do not appear to be involved in osmoregulation, a result in agreement with ultrastructure of these cells (Leader and Green, 1978; Green, 1979; Komnick and Achenbach, 1979; Kohnert *et al*., 2004). Previous investigations regarding the Na^+,K^+-ATPase have shown that their distribution in insects tissues varies between species. It is present in the malpighian tubules, but not in the midgut, of *Drosophila melanogaster* (Linnaeus, 1758) (Lebovitz *et al*., 1989) and in the midgut but not malpighian tubules of the mosquito, *Anopheles stephensi* (Liston, 1901; Emery, 1995).

In conclusion, the basal epithelial cells of the rectal gill lamellae in dragonflies, possess typical ionocytes which are the site of ion pumping, suggested by the presence of a high activity and concentration of Na^+, K^+-ATPase.

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ریزساختار و نقش حفره آبششی لارو سنجاقک (*Libellula lydia*) در عمل تنظیم فشار اسمزی

ص. خدابنده

چکیده

ریزساختار، فعالیت آنزیم Na^+,K^+-ATPase و مکانیابی آن در حفرهٔ آبششی لارو *Libellula lydia* مورد مطالعه قرار گرفت. فعالیت آنزیم Na^+,K^+-ATPase به روش بیوشیمیایی و مکانیابی آن به روش میکروسکوپی ایمنوفلورسانس با استفاده از آنتی بادی $IgG\alpha_5$ انجام گرفت. حفرهٔ آبششی دارای ۶ جفت صفحه آبششی است که در داخل حفرهٔ آبششی بصورت ورقه ورقه روی همدیگر قرار گرفته‌اند. یک لایهٔ بافت پوششی ضخیم و سلولهای چربی تغییر شکل یافته در قسمت پایه هر صفحه آبششی قرارگرفته است. سلولهای بافت پوششی دارای یک لایهٔ کوتیکولی نازک و میکرویلی در ناحیه انتهایی می باشند و این سلولها در قسمت غشاء قاعده‌ای فرورفتگی‌های زیادی دارند که با تعداد زیادی از میتوکندریها مرتبط شده‌اند. سلولهای چربی تغییر شکل یافته، دارای میتوکندری، گلیکوژن و مقدار محدودی قطرات چربی می‌باشند. میزان فعالیت آنزیم Na^+, K^+-ATPase (۱۵/۳۶ µm Pi mg $^{-1}$ protein h^{-1})در ناحیهٔ حفرهٔ آبششی به طور معنی داری بالا بود. روش ایمنوفلورسانس نشان داد که آنزیم Na^+,K^+-ATPase با تراکم بالایی در بخش قاعده‌ای سلولهای پوششی حضور دارد. این یافته‌ها نشان می‌دهد که سلولهای پوششی ناحیهٔ پایه‌ای صفحات آبششی، دارای اختصاصات سلولهای یونوسیت بوده و فعالیت بالا و تراکم زیاد آنزیم Na^+,K^+-ATPase در این سلولها مبین شرکت آنها در عمل تنظیم فشار اسمزی از طریق تبادلات یونی فعال می باشد.

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