

Na⁺,K⁺-ATPase in the Gut of Larvae of the Zygopteran, *Ischnura elegans*, and the Anisoptera, *Libellula lydia*, (Odonata): Activity and Immunocytochemical Localization

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Saber Khodabandeh (2006) Na⁺,K⁺-ATPase in the gut of larvae of the zygopteran, *Ischnura elegans*, and the anisoptera, *Libellula lydia*, (Odonata): activity and immunocytochemical localization. *Zoological Studies* 45(4): 510-516. Na⁺,K⁺-ATPase activity and immunolocalization were demonstrated in the gut of *Ischnura elegans* and *Libellula lydia* larvae. Localization was performed through immunofluorescence light microscopy using the IgGα₅ mouse monoclonal antibody. The Na⁺,K⁺-ATPase activity was significantly higher in the hindgut than in the foregut-midgut in both species. In *I. elegans*, Na⁺,K⁺-ATPase activities were 29.44 and 5.12 μM Pi/mg/protein/h in the hindgut and foregut-midgut, while in *L. lydia*, the activities were 16.24 and 1.98 μM Pi/mg/protein/h in the hindgut and foregut-midgut, respectively. No specific fluorescence staining was observed in the cells of the foregut or midgut regions in either species. Na⁺,K⁺-ATPase was found in the malpighian tubules and rectal pad epithelium in *I. elegans*, and in the epithelium of the basal pads of the rectal gill lamellae in *L. lydia*. A consistently high immunoreactivity was observed in the sides of the lumen of malpighian tubule cells, and a positive and strong fluorescence signal was found in the basolateral sides of the pads of epithelium cells. These findings show that as in crustaceans, this antibody is useful for locating of Na⁺,K⁺-ATPase and ionocytes in insect osmoregulatory tissues. A high concentration of Na⁺,K⁺-ATPase activity in these tissues confirms their participation in osmoregulation through active ion exchange. <http://zoolstud.sinica.edu.tw/Journals/45.4/510.pdf>

Key words: Damselfly, Dragonfly, Immunolocalization, Na⁺,K⁺-ATPase.

The Zygoptera (damselflies) and Anisoptera (dragonflies) are 2 interesting groups of insects because their larvae are fully aquatic while the adults are terrestrial (Corbet 1999). Insect larvae living in fresh water are generally hyperosmotic to their environment. Under this condition, water tends to pass into insects, and they are faced with a continuous osmotic inflow of water and 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 tissues connected to it, such as malpighian tubules (Leader and Green 1978, Nicholls 1983, Zeiske 1992). In general, the gut of insects is composed of 3 major

regions: the foregut, midgut, and hindgut. There is little or no absorption across the foregut, and there is no evidence of the membrane elaboration characteristic of transport in their cells (Chapman 1998). The midgut participates in digestion and absorption of nutrients into the hemocoel. Digestive residues are passed into the hindgut, which also receives fluid from the malpighian tubules. The latter are usually connected to the intestine at the junction between the midgut and hindgut. They remove waste products of cellular metabolism and participate in osmoregulation. The hindgut is divided into a thin ileum and a rectum (Chapman 1998). The rectum of Odonata larvae has been the subject of qualitative anatomical, physiological, histological, and ultrastructural

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investigations (Greven and Rudolph 1973, Wichard and Komnick 1974, Komnick 1978, Green 1979, Komnick and Achenbach 1979, Miller 1994, Kohnert et al. 2004). In damselfly larvae, the rectum possesses 3 epithelial pads which show the excellent structural organization of ion-transporting epithelia (Wichard and Komnick 1974). In dragonfly larvae, the rectum is divided into a large anterior branchial chamber, housing the heavily tracheated respiratory gill lamellae, and a short posterior vestibule (Green 1979, Kohnert et al. 2004).

Na⁺,K⁺-ATPase (sodium-potassium adenosine-triphosphatase) is the plasma membrane-associated enzyme which catalyzes ubiquitous ATP-driven Na⁺/K⁺ transport. This enzyme is crucial to ion and water regulation in fish kidneys (Venturini et al. 1992, Nebel et al. 2005), gills and antennal glands of crustaceans (Lignot et al. 2005, Khodabandeh et al. 2005a,b and 2006), and malpighian tubules of insects (Zeiske 1992, MacVicker et al. 1993, Emery et al. 1998). Na⁺,K⁺-ATPase function and activity have been characterized in some insects and have 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). This enzyme is a heterodimer composed of α - and β -subunits. Mammals have at least 3 α -subunit and 3 β -subunit isoforms (Horisberger et al. 1991). The available data suggest that insects have evolved only 1 isoform of the α -subunit (reviewed by Emery et al. 1998). Recently, immunohistochemical localization of Na⁺,K⁺-ATPase has been recognized as a useful method for locating ionocytes in tissues and organs of crustaceans during embryonic and post-embryonic development (Cieluch et al. 2004, Khodabandeh et al. 2005a and 2006, Lignot et al. 2005).

In insects, although a good amount of information is now available on Na⁺,K⁺-ATPase activity and ion regulatory capacity of the osmoregulatory organs (Maddrell 1977, Green 1979, Emery et al. 1998), the number of investigations on the Odonata is limited. Odonata larvae live in fresh water and undergo various molts before metamorphosing into adults after several years. 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 Achenbach on *Aeshna* larvae (1979). The object of this study was, therefore, to locate ionocytes in the different parts of the gut of the larvae of 2 species of the Odonata by

Na⁺,K⁺-ATPase activity measurement and immunolocalization.

MATERIALS AND METHODS

Na⁺,K⁺-ATPase activity

Na⁺,K⁺-ATPase activities in the different parts of the gut of damselfly and dragonfly larvae were determined according to the technique described by Norby (1988). The foregut-midgut and hindgut samples were quickly excised, weighed, and homogenized separately in cold imidazole buffer (50 mmol/L imidazole, 250 mmol/L sucrose, and 5 mmol/L EDTA at pH 7.4 with HCl).

The cuvette contained 2 ml of the reaction mixture with and without 5 mmol/L ouabain. The composition of the reaction media was 25 mmol/L Tris-HCl, 2 mmol/L MgCl₂, 0.25 mmol/L EGTA (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, and LDH/PK enzymes. Incubation was conducted at 37°C for 30 min, and the Na⁺,K⁺-ATPase reaction was initiated by the addition of homogenate. The Na⁺,K⁺-ATPase activity was expressed as the activity in the presence of ouabain (a specific inhibitor of Na⁺,K⁺-ATPase) subtracted from the activity obtained in its absence. Results are expressed in terms of both milligrams of tissue (wet weight) and milligrams of protein. Protein concentrations were determined according to the modified procedure described by Lowry et al. (1951).

Statistical treatment of the data

Na⁺,K⁺-ATPase activity data are presented as the mean \pm SD. Paired Student's *t*-test was used to determine the significant difference ($p < 0.05$) as the confidence limit.

Na⁺,K⁺-ATPase immunolocalization

Immunolocalization of Na⁺,K⁺-ATPase was performed through immunofluorescence light microscopy using a mouse monoclonal antibody, IgG α_5 , raised against the α -subunit of chicken Na⁺,K⁺-ATPase (Takeyasu et al. 1988) obtained from the Development Studies Hybridoma Bank, developed under the auspices of the NICHD and maintained by the University of Iowa (USA). The α_5 monoclonal antibody recognizes all 3 isoforms of the α subunit of Na⁺,K⁺-ATPase in invertebrates,

where they are present (Kone et al. 1991). This antibody is able to cross-react with the α subunit of invertebrate Na^+, K^+ -ATPase (Baumann et al. 1994, Just and Walz 1994). Following 24 h 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. Sections were preincubated for 10 min in 0.01 mM Tween 20 and 150 mM NaCl in 10 mM phosphate buffer, pH 7.3, and then treated with 50 mM NH_4Cl in phosphate-buffered saline (PBS), pH 7.3, for 5 min to mask the free aldehyde groups of the fixative. Sections were washed in PBS and incubated for 10 min 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 $\mu\text{g}/\text{ml}$ was placed on the sections and incubated for 2 h at room temperature in a moist chamber. The sections were then incubated for 1 h in the secondary antibody (fluorescein isothiocyanate conjugate, FITC) in dark conditions. The slides were rinsed in BS and mounted in a medium for fluorescent microscopy (Sigma, ref. 7534) to retard photobleaching. Negative control sections were incubated in BSA-PBS without the primary antibody. Sections were examined with a fluorescence microscope (Leitz Diaplan coupled to a Ploemopak 1-Lambda lamp) with the appropriate filter set (filters of 450-490 nm).

RESULTS

Na^+, K^+ -ATPase activity

Results of the biochemical assay for Na^+, K^+ -ATPase activity are illustrated in table 1. The Na^+, K^+ -ATPase activity was significantly higher in the hindgut than foregut-midgut in both species (Figs. 1, 2).

In *I. elegans*, Na^+, K^+ -ATPase activities were 29.44 and 5.12 $\mu\text{M Pi}/\text{mg}/\text{protein}/\text{h}$ in the hindgut

and foregut-midgut, respectively (Fig. 1). In *L. lydia*, Na^+, K^+ -ATPase activities were 16.24 and 1.98 $\mu\text{M Pi}/\text{mg}/\text{protein}/\text{h}$ in the hindgut and foregut-midgut, respectively (Fig. 1). The Na^+, K^+ -ATPase activity, expressed in units per gram of tissue was also significantly higher in the hindgut than foregut-midgut in these species (Table 1, Fig. 2).

Immunolocalization of Na^+, K^+ -ATPase

Immunofluorescence microscopy showed consistent results within different regions of the gut (Fig. 3D-F, 3H-I). Positive control crayfish antennal glands were consistently brightly immunostained (not shown). In thin sections, negative controls showed no specific binding within the gut epithelium (Fig. 3A). In *I. elegans*, no specific fluorescence staining was observed in cells of the foregut (Fig. 3B) or midgut regions (Fig. 3C). The rectal wall contains 3 epithelial pads (Fig. 3A, D). In cross-section, each pad was lined by 15-20 columnar cells (with an average high of 65 μm). Malpighian tubule sections were observable

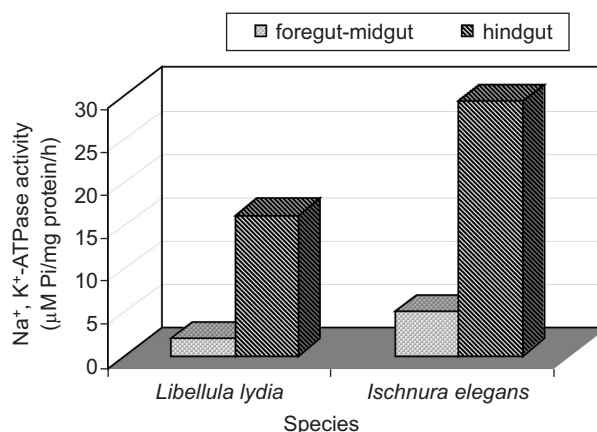


Fig. 1. Variation of Na^+, K^+ -ATPase activity ($\mu\text{M Pi}/\text{mg}/\text{protein}/\text{h}$) in the foregut-midgut and in the hindgut of damselfly *Ischnura elegans*, and dragonfly *Libellula lydia* larvae.

Table 1. Na^+, K^+ -ATPase activity in the foregut-midgut and hindgut of damselfly, *Ischnura elegans*, and dragonfly, *Libellula lydia*, larvae (mean \pm SD)

Species	Tissue	Replicates	Na^+, K^+ -ATPase activity ($\mu\text{M Pi}/\text{mg}/\text{protein}/\text{h}$)	
			per mg tissue (wet wt)	per mg protein
<i>Libellula lydia</i>	Foregut-midgut	7	0.17 \pm 0.03	1.98 \pm 0.30
	hindgut	6	0.92 \pm 0.12	16.24 \pm 2.30
<i>Ischnura elegans</i>	Foregut-midgut	5	0.48 \pm 0.07	5.12 \pm 0.62
	hindgut	7	4.49 \pm 1.11	29.44 \pm 3.82

around the rectal pads (Fig. 3A, D). Na⁺,K⁺-ATPase was detected in the rectal pad epithelium and in the malpighian tubules (Fig. 3D-F). In the rectal epithelial pads, the fluorescence was concentrated on the basolateral side of the cells (Fig. 3F). A consistently high immunoreactivity was also observed in the sides of the lumen of malpighian tubule cells (Fig. 3E). In *L. lydia*, no specific fluorescence staining was observed in the cells of the foregut (not shown) or the midgut (Fig. 3G). The anterior portion of the rectum is modified into a branchial chamber. Autofluorescence was observed on the cuticle (Fig. 3H). The rectal branchial chamber consists of longitudinally oriented curtain-like folds that extend into the rectal lumen from 6 pairs of gill lamellae (Fig. 3H). In cross-section, a thickened epithelial layer and a modified fat body cell layer were present at the base of each gill lamella (Fig. 3I). Strong, positive Na⁺,K⁺-ATPase fluorescence was found on the basolateral sides of the basal epithelial cells of the gill lamellae (Fig. 3H, I). No immunofluorescence was detected in the gill lamellae or basal fat body cells (Fig. 3I).

DISCUSSION

Na⁺,K⁺-ATPase provides at least part of the driving force for transepithelial movement of monovalent ions across transporting tissues in many aquatic animals including invertebrates. In crustaceans, the α_5 monoclonal antibody has previously been used in the study of the branchial cavity (Lignot et al. 2001), antennal glands, and intestine (Khodabandeh et al. 2005a, b and 2006) of

Homarus gammarus and the gills (Lignot et al. 2005) and antennal glands (Khodabandeh et al. 2005a) of *Astacus leptodactylus*.

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 organs 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 2006). This is the first study to demonstrate Na⁺,K⁺-ATPase activity in insect osmoregulatory tissues, and the first to use the IgG α_5 mouse monoclonal antibody (raised against the α -subunit of chicken Na⁺,K⁺-ATPase) for immunolocalization of this enzyme. The present results revealed that Na⁺,K⁺-ATPase activity was significantly higher in the hindgut than foregut-midgut in both the zygopteran (*I. elegans*) and anisopteran (*L. lydia*). In the immunocytochemical study, the mouse monoclonal IgG α_5 appeared to recognize its epitope in the malpighian tubules and rectal pad epithelium of the hindgut in *I. elegans*, and in the basal epithelial cells of the rectal gill lamellae in *L. lydia*. Thus, Na⁺,K⁺-ATPase activities are present in the hindgut including malpighian tubules and rectal pad epithelium Na⁺,K⁺-ATPase in *I. elegans*, and basal epithelial cells of the rectal gill lamellae in *L. lydia*. With Na⁺,K⁺-ATPase immunolocalization, the high antibody specificity may be attributable to conservation of the α -subunit of the protein in the animal kingdom during the course of evolution, as illustrated by analysis of the amino acid sequence of *Artemia franciscana*, which is about 80% similar to the vertebrate sequence (Macias et al. 1991). Ultrastructurally, epithelial pad cells in the rectum of zygopteran larvae and basal epithelial cells of the gill lamellae in anisopteran larvae exhibit ion-transporting epithelial characteristics, such as extensive basal membrane infolding associated with more-numerous mitochondria and apical microvilli which simultaneously provide extracellular channels. From the combination of observations conducted by electron microscopy (Wichard and Komnick 1974, Green 1979, Komnick and Achenbach 1979), Na⁺,K⁺-ATPase activity, and immunolocalization (this study), I concluded that the epithelial pads of the rectum in *I. elegans* larvae, and the basal epithelial of the gill lamellae in *L. lydia* larvae are lined by ionocytes (chloride cells) which are the site of ion pumping. The presence of high levels of Na⁺,K⁺-ATPase activity and strong immunoreactivity sug-

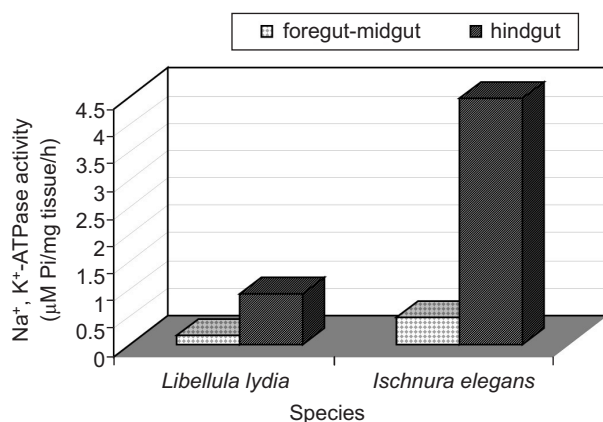


Fig. 2. Variation of Na⁺,K⁺-ATPase activity, expressed in unite per g of tissue, in the foregut-midgut and in the hindgut of damselfly *Ischnura elegans*, and dragonfly *Libellula lydia* larvae.

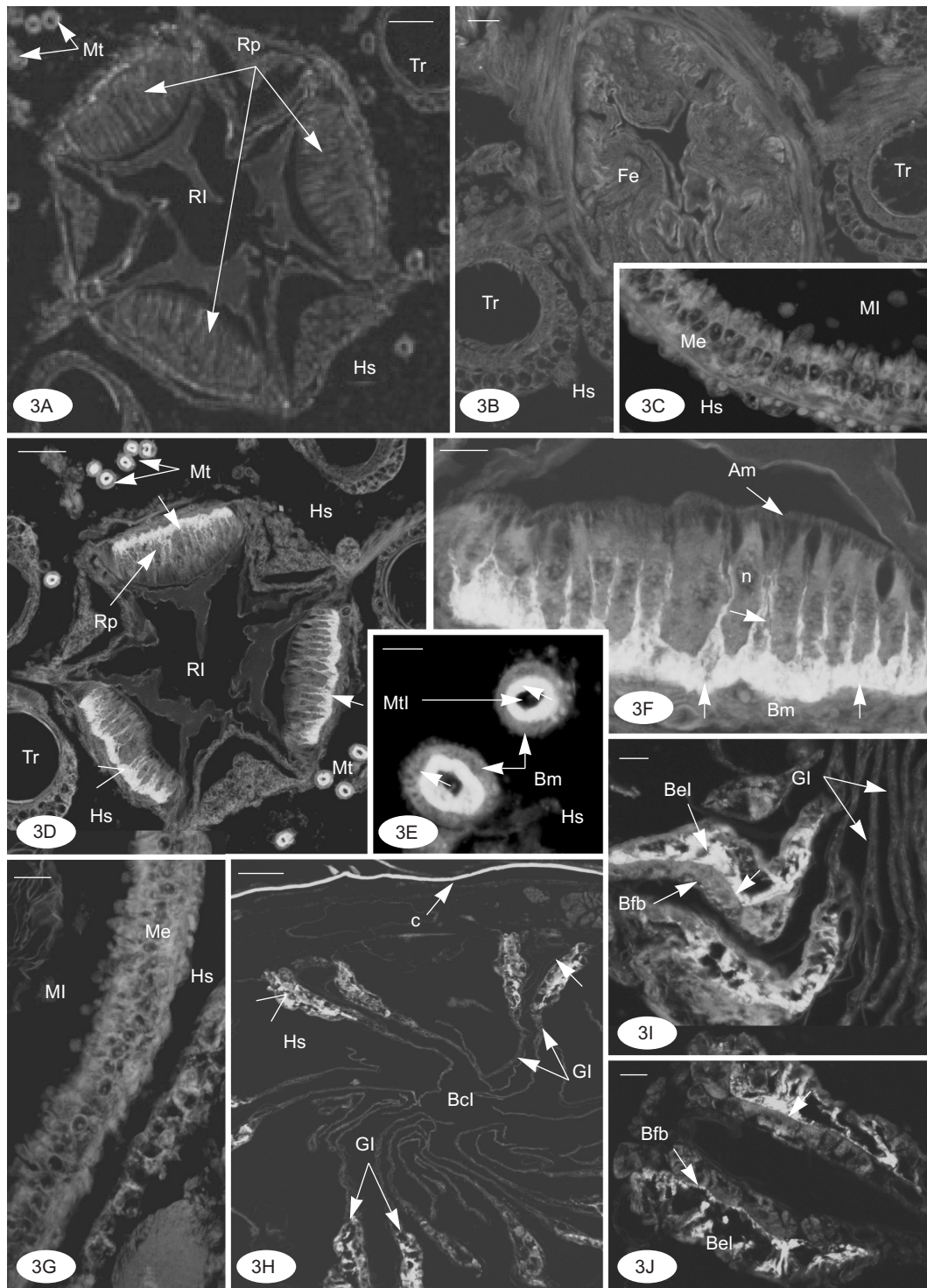


Fig. 3. Immunolocalization of Na^+, K^+ -ATPase in the gut of damselfly, *Ischnura elegans*, and dragonfly, *Libellula lydia*, larvae. (A-F) *Ischnura elegans*. (A) Negative control: hindgut (rectal pad epithelium); (B) foregut; (C) midgut; (D) hindgut (rectal pad epithelium); (E) malpighian tubule sections; (F) rectal pad epithelial cells. (G-J) *Libellula lydia*. (G) Midgut; (H) branchial chamber section; (I, J) basal thickened regions of the gill lamellae. Free arrows indicate the immunofluorescent activity of Na^+, K^+ -ATPase. Am, apical microvilli; Bcl, branchial chamber lumen; Bel, basal epithelial layer; Bfb, basal fat body; Bm, basal membrane; C, cuticle; Fe, foregut epithelium; GI, gill lamellae; Hs, hemolymph space; Me, midgut epithelium; MI, midgut lumen; Mt, malpighian tubule; Mtl, malpighian tubule lumen; N, nucleus; RI, rectal lumen; Rp, rectal pad; Tr, trachea. Bars: 45 μm (A, B), 10 μm (C), 90 μm (D), 15 μm (E), 18 μm (F), 10 μm (G), 100 μm (H), 30 μm (I, J).

gest an active transport in these cells. This permits larvae to cope with the low osmolality of fresh water through hyperosmoregulation. This result is in agreement with results of Phillips and Audsley (1995) who showed that Na⁺,K⁺-ATPase is abundant in the lateral membranes of rectal pads in the hindgut of the locust, *Schistocera gregarina*. The biochemical chloride cell localization indicates that these cells are also able to accumulate chloride ions on 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 2006, Lignot et al. 2005).

I detected no trace of Na⁺,K⁺-ATPase in the basal fat body cells of the *L. lydia* gill chamber, and they do not appear to be involved in osmoregulation, a result in agreement with the ultrastructure of these cells (Leader and Green 1978, Green 1979, Komnick and Achenbach 1979).

The Na⁺,K⁺-ATPase activity in the foregut-midgut of both species was comparatively very weak, corresponding only to baseline levels, a result in agreement with immunolocalization results. I observed that in *I. elegans*, some of malpighian tubules were present in the midgut region. Note that the Na⁺,K⁺-ATPase activity in the foregut-midgut of *I. elegans* also included the activity of malpighian tubules. Na⁺,K⁺-ATPase activity is probably diluted by the presence of the respiratory gill lamellae and basal fat body cells in the hindgut of *L. lydia*. Previous investigations regarding Na⁺,K⁺-ATPase showed that its distribution in insect tissues varied among species. It is present in malpighian tubules, but not the midgut, of *Drosophila melanogaster* (Lebovitz et al. 1989) and in the midgut, but not malpighian tubules, of the mosquito, *Anopheles stephensi* (Emery et al. 1998).

In conclusion, as in fish and crustaceans, the IgG α_5 mouse monoclonal antibody cross-reacts with Na⁺,K⁺-ATPase of insect cation-transporting plasma membranes of osmoregulatory cells (ionocytes). The rectal epithelial pads of the hindgut in a damselfly and the basal epithelial cells of the rectal gill lamellae in a dragonfly possess typical ionocytes which are the sites of ion pumping, suggested by the presence of a high concentration of Na⁺,K⁺-ATPase.

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