SYSTEMATICS, MORPHOLOGY AND PHYSIOLOGY

Analysis of Nucleus Activity in Malpighian Tubules of *Diatraea saccharalis* (Fabricius) (Lepidoptera: Crambidae) Larvae by Critical Electrolyte Concentration

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ABSTRACT - *Diatraea saccharalis* (Fabricius) has cryptonephridial type Malphigian tubules (MT). This type of MT is characterized by the penetration of the distal part of the MT into the external walls of the rectum, which is usually lined with a perinephral membrane. The MT is divided into three differentiated regions: proximal, middle and distal. In this study, our objective was to compare the nuclear activities of each one of the three regions of the *D. saccharalis* MT by using a nuclear basophilic technique and critical electrolyte concentration with a toluidine blue stain at pH 4.0. This method allows differentiation of DNA/protein complexes in *in situ* and *in vitro* chromatin. MT chromatin structure in *D. saccharalis* is variable. Fifth instars have a more decondensed chromatin than fourth instars. The distal tubule region was the most decondensed region of the MT. Our data show an elevated genetic activity of the MT in the pre-metamorphosis period. The distal region of the MT has the highest observed activity, which may be associated with the re-absorption of useful components and the excretion of waste materials.

KEY WORDS: Cytochemistry, larval development, cryptonephridial system

Malpighian tubules (MT) are the main excretory organs of insects and are responsible for maintaining organism homeostasis (Caetano 1988, Maddrell & O'Donnell 1992, Chapman 1998, O'Donnell & Spring 2000). The excretory systems of Coleoptera and Lepidoptera larvae follow a cryptonephridial arrangement in which the MT distal ends do not flow freely into the hemocel but adhere to the rectum instead. The MT are separated from the rectum lumen by a perinephridial gap. The structure is lined by a poorly permeable perinephral membrane that constitutes the cryptonephridial complex (Díaz *et al* 2000, Liao *et al* 2000, Levy *et al* 2004).

The MT of *Diatraea saccharalis* (Fabricius) have three distinct regions during the larval stage: the proximal, the middle and the distal or cryptonephridial regions. The formation of the cryptonephridial system occurs at the distal portion of the tubules, similar to a sheath that involves the final portion of the rectum. The distal region, isolated by the perinephral membrane, maintains itself close to the rectal epithelium (Rigoni *et al* 2004).

The toluidine blue (TB) solution at pH 3.6-4.0 has an affinity for the phosphate groups available in DNA and RNA. The chromatin of somatic cells treated with TB generally show violet-colored metachromatic basophilia. Metachromasy occurs when TB molecules, linked to phosphate groups of nucleic acids, are piled up and interact among themselves (Mello 1997). If TB staining occurs in the presence of Mg^{2+} ions at determined concentrations, metachromasy of the nucleic acid will not occur. This phenomenon has been called critical electrolyte concentration (CEC) and is visually recognized by its greenish chromatin color (Vidal & Mello 1989).

CEC research with TB staining and Mg^{2+} as an inorganic ion, which was originally used for *in vitro* protein-DNA models (Vidal & Mello 1989), has been efficiently used to differentiate types of protein-DNA complexes in *in situ* chromatin (Mello & Falco 1996, Mello 1997, Falco & Mello 1999). Under different conditions of stress, physiology and development, there is a variation in the competition between TB and Mg^{2+} for linking sites in chromatin when heterochromatin and euchromatin are compared. Differences in the availability of phosphate groups in nucleic acid, and the packaging of the nucleic acid, determine the degree of condensation and changes in CEC values. CEC values are higher in condensed than in decondensed chromatin (Mello 1997, Monteiro & Mello 1998, Falco & Mello 1999). Chromatin condenses and decondenses proportionally to the rate at which DNA specific cell sequences are accessed for gene transcription (Alberts *et al* 2008).

In this study, we analyzed the chromatin structure and the gene activity in the three morphologically distinct regions of the larval Malpighian tubules of *Diatraea saccharalis* (Fabricius) by using nuclear basophilia and critical electrolyte concentration techniques with TB staining, coupled to $MgCl_2$ at different concentrations (Vidal & Mello 1989, Mello 1997).

Material and Methods

Insect rearing and Malpighian tubules dissection. Larvae of *D. saccharalis* were maintained in glass tubes and fed on an artificial diet (Hensley & Hammond 1968) under controlled conditions (25 ± 1ºC, 70 ± 10% RH, and 14h photoperiod). Malpighian tubules were dissected from 4th and 5th instars of *D. saccharalis* in an insect saline (1.80% NaCl, 1.88% KCl, 0.16% CaCl, 0.004% $NaHCO_3$) under a stereomicroscope.

Nuclear basophily and critical electrolyte concentration (CEC). Dissected MT of 4th and 5th instars of *D. saccharalis* were crushed between a slide and a cover slide in 45% acetic acid and frozen in liquid nitrogen. Samples were taken from the liquid nitrogen, left to stand at room temperature and the cover slide was removed once samples had defrosted. Samples were then fixed in ethanol: acetic acid (3:1 v/v) for 2 min, washed in 70% ethanol for 10 min, and stained with 0.025% TB in McIlvaine buffer (12.9 g citric acid and 10.9 g dibasic sodium phosphate in 1000 ml distilled and deionized water q.s.q) at pH 4.0, without $MgCl_2$ for 20 min. Samples were also stained with 0.025% TB in McIlvaine buffer (pH 4.0) containing different concentrations of $MgCl_2$ (0.02, 0.05, 0.08, 0.10, 0.12, 0.15, 0.20, and 0.30 mol/l). After staining, samples were washed in deionized water (five seconds) and then air dried. Xylol bleaching was undertaken for 15 min and samples were slide-mounted in Entellan (Vidal & Mello 1989). Once the mounting agent had dried, slides were analyzed under a standard light microscope (Carl Zeiss, Jena, Germany) and photographed using an Aiptek 3.1 mega pixels digital camera (AIPTEK Inc., China, 2003).

Histological analysis. MT of 4th and 5th instars of *D. saccharalis* were fixed, dehydrated in an ethanol series, followed by ethanol/xylol (1:1 v/v) and xylol treatments, and embedded in paraffin. Embedded samples were subjected to microtomy and 8-µm-thick sections were taken serially. Sections were slide-mounted, rehydrated with xylol, ethanol/xylol (1:1 v/v) and a graded series of ethanol (100%, 95%, 90%, 80%, 70%), stained with hematoxylin and eosin and assembled on semi-permanent slides, selected and photographed.

Scanning electron microscopy. MT were fixed according to Karnovsky (1965), dehydrated in a graded series of acetone and critical point dried in a Balzers CPD/030 critical point dryer. Samples were placed on stubs and gold-sputtered with 10 nm of gold. Specimens were examined and photographed on a JEOL 15 SM-P 15 scanning electronic microscope.

Results

The principal cell of the three morphologically similar regions in the two instars analyzed showed a large nucleus, with highly de-condensed chromatin and a central nucleolus with metachromatic basophilia (Fig 1).

Principal cells of the MT of 4th instars presented a CEC

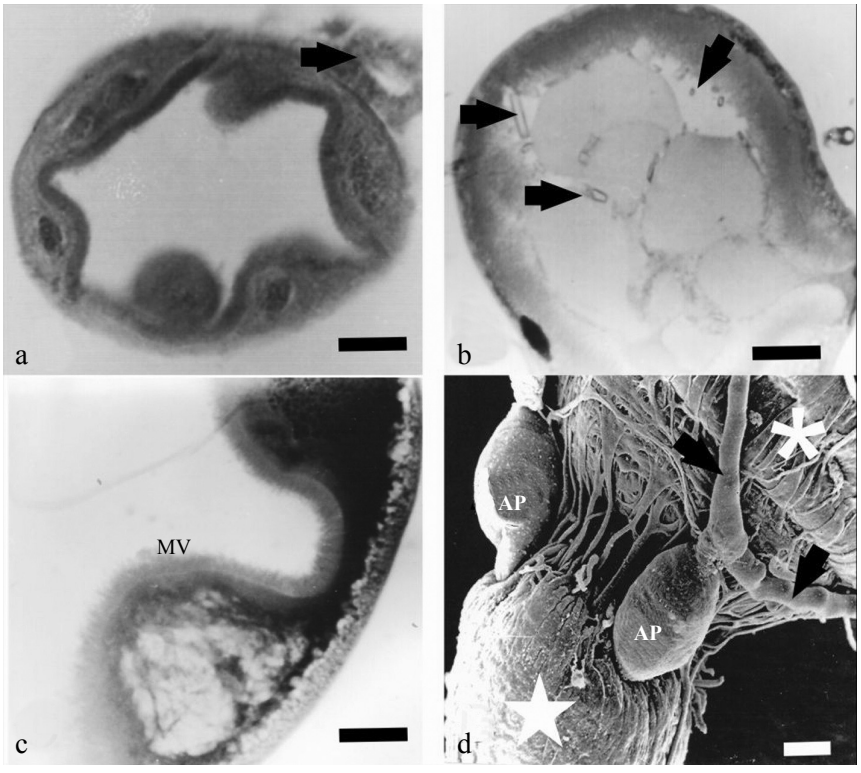

Fig 1 *Diatraea saccharalis*. a) A cross-sectioned Malpighian tubule. Arrow shows a tracheole (bar = 30 µm); b) Crystals (arrows) contained in the lumen of a Malpighian tubule (bar = 30 µm); c) LM of the microvilli (MV) at the internal cell surface of a Malpighian tubule (bar = 30 µm); d) SEM of the ampulla (AP) of a MT. Arrows show the proximal region of MT. * Indicates mesenteron. ☆ Indicates hindgut (bar = 100 µm).

Table 1 Nuclear basophilia and critical electrolyte concentration (CEC) in the chromatin of Malpighian tubules dissected from late 4th and 5th instars of *Diatraea saccharalis* after treatment with 0.025% TB in McIlvaine buffer (12.9 g citric acid and 10.9 g dibasic sodium phosphate in distilled and deionized water 1000 ml q.s.q) at pH 4.0 added with different concentrations of $MgCl_2$.

Toluidine blue (TB) + $MgCl_2$ (Mol/l)	4th instar			5th instar		
	Proximal region	Middle region	Distal or cryptonephridia region l	Proximal region	Middle region	Distal or cryptonephridia region l
TB absent of $MgCl_2$	Violet	Violet	Violet	Violet	Violet	Violet
TB + 0.02	Violet/blue	Violet	Violet	Violet/blue	Violet	Violet
TB + 0.05	Violet/blue	Blue	Blue	Violet/blue	Violet/blue	Blue
TB + 0.08	Blue	Blue	Blue	Blue	Blue/green	**Green (CEC)**
TB + 0.10	Blue	Blue/green	Blue/green	**Green (CEC)**	**Green (CEC)**	Blue
TB + 0.12	**Green (CEC)**	**Green (CEC)**	**Green (CEC)**	Blue/green	Violet	Blue
TB + 0.15	Blue/green	Blue/green	Blue/green	Blue	Blue	Violet/blue
TB + 0.20	Blue/green	Blue/green	Blue/green	Blue	Blue	Blue
TB + 0.30	Blue/green	Blue/green	Blue/green	Green	Green	Blue/green

value (metachromasy abolishment) at 0.12mol/l in all of the three regions analyzed. In 5th instars, cells had a different basophilic response (with the exception of TB without $MgCl_2$). The proximal and middle regions showed a CEC value of 0.10 mol/l of $MgCl_2$, whereas the distal region had a value of 0.08 mol/l of $MgCl_2$ (Table 1). Scanning electron and light microscopy indicated the presence of trachea and tracheoles (Fig 1) in all regions of the tubule, but mostly in the middle and distal regions.

Histological analysis showed that cells of the three regions of the Malpighian tubules of the sugarcane borer are quite similar, with all of them displaying microvilli and crystals in the lumen (Fig 1), mainly at the distal region.

Discussion

CEC results (represented by mol/l of $MgCl_2$) are characterized by the abolishment of the nuclear metacromasy and are affected by chromatin organization and structure. Condensed chromatin needs more $MgCl_2$ to abolish metachromasy and show higher CEC values than those of de-condensed chromatin (Taboga *et al* 1996, Mello 1997).

The packaging of eucaryotic DNA into chromatin provides many opportunities for transcriptional regulation. Mechanisms that create different chromatin structures in different regions of a cell's genome are used to control many genes in eucaryotes (Alberts *et al* 2008).

CEC values can indicate how metabolically active an organ is, as shown by the low values observed for the MT of *Triatoma infestans* Klug (Mello & Vidal 1989) and *Apis mellifera* L (Mello & Falco 1996), as compared to that of several other species (Falco & Mello 1999, Falco *et al* 1999).

According to Liao *et al* (2000), the composition and the volume of insect hemolymph are determined by the MT which produce urine, eliminate excess salt and nitrogenated products and re-absorb contents benefiting the organism (Wigglesworth 1974, Rigoni *et al* 2004). MT may exceptionally have a secretory function, besides excretion, during the cocoon stage. They may function not only as salivary and collateral glands (Wigglesworth 1974), but may even detoxificate the organism from pesticides (McGettigan *et al* 2005). Ramsay (1976) stated that the cryptonephridial system aids in the concentration of ions in the final stage of development in Lepidoptera.

A comparison of the two larval instars analyzed in *D. saccharalis*, using CEC and nuclear basophilia methods shows the most compact form of chromatin in the MT principal cells of young larvae. Consequently, there is a lower gene activity and a lower synthesis of polypeptides involved in excretion and re-absorption. It may be concluded that during the last instar, the distal region has the most decondensed chromatin structure and the highest gene activity, suggesting that this region is metabolically more active than that of the proximal and middle regions.

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