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Biochemical activity and multiple locations of particulate guanylate cyclase in *Rhyacophila dorsalis acutidens* (Insecta: Trichoptera) provide insights into the cGMP signalling pathway in Malpighian tubules

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ABSTRACT

In insect renal physiology, cGMP and cAMP have important regulatory roles. In *Drosophila melanogaster*, considered a good model for molecular physiology studies, and in other insects, cGMP and cAMP act as signalling molecules in the Malpighian tubules (MTs).

However, many questions related to cyclic nucleotide functions are unsolved in principal cells (PC) and stellate cells (SC), the two cell types that compose the MT. In PC, despite the large body of information available on soluble guanylate cyclase (sGC) in the cGMP pathway, the functional circuit of particulate guanylate cyclase (pGC) remains obscure. In SC, on the other side, the synthesis and physiological role of the cGMP are still unknown. Our biochemical data regarding the presence of cyclic nucleotides in the MTs of *Rhyacophila dorsalis acutidens* revealed a cGMP level above the 50%, in comparison with the cAMP. The specific activity values for the membrane-bound guanylate cyclase were also recorded, implying that, besides the sGC, pGC is a physiologically relevant source of cGMP in MTs. Cytochemical studies showed ultrastructurally that there was a great deal of pGC on the basolateral membranes of both the principal and stellate cells. In addition, pGC was also detected in the contact zone between the two cell types and in the apical microvillar region of the stellate cells bordering the tubule lumen. The pGC signal is so well represented in PC and, unexpectedly in SC of MTs, that it is possible to hypothesize the existence of still uncharacterized physiological processes regulated by the pGC-cGMP system.

1. Introduction

In Insecta, the Malpighian tubule (MT) is a fluid-transporting organ that filters the haemolymph and secretes a liquid that is comparable to primary urine of vertebrates. Besides their role in osmoregulation, MTs are highly specialized in organic solute transport, metabolism and detoxification (Dow and Davies, 2001, 2006).

In many insect groups, the MTs have morphologically and functionally distinct segments and consist of two cell-types, the principal cells (PC) and the stellate cells (SC) (Sozen et al., 1997). Characterized by different functions and distribution along the tubule, these epithelial cells are fundamental to insect renal physiology. In general, the PC are known to produce primary urine and to actively transport ions, while the SC are believed to be the route for Cl^- and water movement into the lumen (Dow and Davies, 2001).

In many insects the fluid secretion by the MTs is stimulated by diuretic hormones such as corticotropin releasing factor-related hormone, calcitonin-related diuretic hormone, leucokinins and a large number of neuropeptides (Terhzaz et al., 1999; Dow and Davies, 2001). In contrast to the large number of diuretic factors known to stimulate MT secretion, only a few antidiuretic factors that inhibit MT secretion have been identified (Quinlan et al., 1997; Paluzzi et al., 2008).

Along with the research on vertebrate osmoregulation, studies of the hormonal influence on insect fluid secretion by the second messenger control systems have received increasing attention.

In the principal cells of the *Drosophila melanogaster* MTs, both cAMP and cGMP stimulate the vacuolar H^+-motive ATPase located in the apical membrane to induce fluid secretion (Beyenbach, 2001, 2003a). The effects of the cyclic nucleotides on the chloride flux

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through Cl^- channels in the stellate cells (O'Donnell et al., 1998, 2003) and on the water flux through aquaporins, are still being studied (Kaufmann et al., 2005; Spring et al., 2009).

In the MT principal cells, the calcitonin-like diuretic hormone stimulates the cAMP production and increases the transepithelial voltage (Coast et al., 2001). In contrast, the CAPA-1 and CAPA-2 in the principal cells of *D. melanogaster* stimulate MT epithelial fluid transport via the cGMP signalling pathway (Davies et al., 1995; Kean et al., 2002); this also occurs via their homolog, the CAP_{2b}, in the tobacco hornworm moth, *Manduca sexta* (Huesmann et al., 1995).

The cGMP signalling occurs in CAP_{2b} through an increase in intracellular cell calcium (Rosay et al., 1997) which activates nitric oxide synthase (NOS) an enzyme found exclusively in the principal cell (Dow et al., 1994; Davies et al., 1997; Davies, 2000).

Using *D. melanogaster* tubules, it has been shown that exogenous cGMP directly increases the rate of fluid transport (MacPherson et al., 2004). This cGMP enters tubule cells via a cyclic nucleotide transporter (Riegel et al., 1998; Sager, 2004), probably to reach its cellular targets such as cyclic nucleotide-gated channels, cGMP-regulated phosphodiesterases and cGMP-dependent protein kinases (Munzel et al., 2003; Day et al., 2006; Davies, 2006).

Furthermore, the excretion of cGMP is also a regulatory event. Studies on cGMP efflux in urine have demonstrated that tubular principal cells also extrude this cyclic nucleotide through an active ATP-dependent transport that involves membrane proteins (Day et al., 2006; Evans et al., 2008).

Thus, many data show that signalling via cGMP is involved in important regulatory processes that affect the principal cell. However, to our knowledge, there is no evidence to date in support of a role for cGMP in the stellate cell.

The synthesis of cGMP from guanosine triphosphate (GTP) is catalyzed by guanylate cyclase (GC), which is widely present in the animal world. This enzyme has two isoforms: soluble GC (sGC) and particulate GC (pGC). The particulate forms of GC comprise an extracellular ligand-binding domain and a membrane-spanning domain, and they can function as receptors (Lucas et al., 2000). The demonstration that sGC of the principal cell can be activated by nitric oxide, thus providing the synthesis of cGMP and, subsequently, the tubular flux modulation (Rosay et al., 1997; Davies et al., 1997; Davies, 2000; Kean et al., 2002; McGettigan et al., 2005), was a great contribution in physiological studies on the cGMP signalling pathway in MT. While the molecular pathway of sGC is well documented, a functional pathway of pGC in insect MTs is not known. Recently, an interesting work reported that the manipulation of cGMP via mammalian pGC transgene expressed in either principal or stellate cells elevates fluid transport (Kerr et al., 2004).

Great interest was aroused when vertebrate pGC was shown to be part of a membrane receptor that is sensitive to molecules belonging to the family of natriuretic factors (Waldman et al., 1984; Kuno et al., 1986; Chinkers et al., 1989). Most of the biological activities of the atrial natriuretic peptides (ANP) are thought to be mediated by guanylate cyclase-coupled receptor A and receptor B (Suga et al., 1992), which are widely distributed in the target tissues as well as in organs without a specific osmoregulatory function (Vagnetti et al., 1995; Kuhn, 2004).

The particulate guanylate cyclases are also integrated in the cell surface receptors for guanylin and uroguanylin. Abundant in vertebrates, these endogenous peptides also stimulate cGMP production and promote fluid and electrolyte secretion in renal and intestinal target cells (Forte et al., 2000; Forte, 2005; Sindic and Schlatter, 2006).

The highly conserved transductive strategies, found extensively in vertebrates, may also exist in insect physiology where they resolve analogous osmoregulatory problems.

Thus, to better understand the physiological analogies between two phylogenetically distant animal groups, Insects and Vertebrates, we investigated the second messenger system of the cGMP and, for a comparative purpose, the cAMP system in Malpighian tubules of adult *Rhyacophila dorsalis acutidens* (Insecta: Trichoptera), an insect that has been extensively studied in our laboratories (Bicchierai et al., 1997; Bicchierai and Corallini, 2005). The morphological characteristics of the principal and stellate cells in trichopteran MT are in accord with those described in the MT of the dipteran *Drosophila*, which is considered an organotypic model for comparative studies in renal physiology (Davies, 2006). In this study, the MT pGC was investigated with specific attention, in order to explore the physiological significance of the local generation of cGMP in the principal cells and especially in the lesser known stellate cells.

2. Materials and methods

2.1. Animals

Adult of *Rhyacophila dorsalis acutidens* (Insecta: Trichoptera) were taken upon emergence from pupae which were collected in the River Nestore (Perugia, Italy) and kept in the laboratory at room temperature. Specimens were cooled on ice before the dissection procedures. Some specimens were used to prepare the Malpighian tubules for microscopic observation.

2.2. Measurement of cGMP and cAMP levels

Three samples, 10 specimens each, were used. The Malpighian tubules were rapidly excised from the specimens, frozen in liquid N_2 to prevent enzymatic degradation of the cyclic nucleotides, suspended in a 5-fold volume (w/v) of 6% cold perchloric acid and then homogenized. The homogenates were boiled for 5 min and centrifuged at 15,000 × *g* for 10 min, at 4 °C. Supernatants were then neutralized and then lyophilized. The samples, resuspended in TRIS/EDTA buffer (50 mM Tris/HCl, 4 mM EDTA, pH 7.5), were assayed for cyclic GMP and AMP by a radioimmunoassay kit (TRK 500, RIA kit for cGMP and TRK 432, RIA kit for cAMP; Amersham, UK), following the manufacturer's instructions (Steiner et al., 1972). The radioactivity was measured using a scintillation counter (Beckman, High Wycombe, UK). Values were expressed as pmol cyclic nucleotide per mg of perchloric precipitable protein. Determination of protein content was performed as described by Lowry et al. (1951), using bovine albumin as the standard.

2.3. Guanylate and adenylate cyclase specific activities

Malpighian tubules from a pool of 30 adult subjects were isolated and immersed in *Drosophila* saline medium prior to homogenization. Homogenization was performed in ice-cold 10 mM Tris–HCl, pH 7.6, at 4 °C, and homogenates were filtered through a thick gauze.

A fraction of filtered homogenates was centrifuged for 60 min at 105,000 × *g* and the supernatants were collected as the source of the sGC. In order to remove the soluble GC from the particulate GC, the pellet was resuspended in 10 mM Tris–HCl, again homogenized and centrifuged for 60 min at 105,000 × *g* at 4 °C. The final pellet was resuspended in the same buffer and assayed for particulate guanylate cyclase activity. The assay for both soluble and particulate forms of guanylate cyclase, was performed using unlabelled GTP as substrate and the reaction buffer contained 50 mM Tris–HCl buffer (pH 7.6), 2 mM IBMX, 15 mM creatine phosphate, 8 μg/100 μl creatine kinase (120–135 units/mg), 1 mM GTP and 4 mM $MnCl_2$, according to Garbers and Murad (1979).