INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

THE PHYSIOLOGICAL SIGNIFICANCE OF PUTATIVE DIURETIC FACTORS IN THE YELLOW FEVER MOSQUITO, *AEDES AEGYPTI*

by

Craig Cady

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF ENTOMOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

1997

THE UNIVERSITY OF ARIZONA ®
GRADUATE COLLEGE

As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Craig John Cady

entitled The Physiological Significance of Putative Diuretic Factors in the Yellow Fever Mosquite, Aedes aegypti

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

R. F. Chapman Reginald Chapman	3/17/97 Date
Eldon J. Braun Eldon Braun	3/17/97 Date
Henry Hagedorn Henry Hagedorn	3/17/97 Date
Timothy Bradley Timothy Bradley	3/17/97 Date
	Date

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Henry Hagedorn Dissertation Director Henry Hagedorn	3/17/97 Date

STATEMENT BY AUTHOR

This dissertation has been submitted in partial fulfillment of requirements for an advanced degree at The University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the Library.

Brief quotations from this dissertation are allowable without special permission, provided that accurate acknowledgment of source is made. Requests for permission for extended quotation form or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the Graduate College when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

SIGNED: 

ACKNOWLEDGMENTS

I wish to express my gratitude to my committee members; Dr. Reg Chapman, Dr. Timothy Bradley, Dr. Eldon Braun and Dr. Jose Ribeiro. I thank my advisor and mentor Dr. Henry H. Hagedorn for his guidance, friendship and patience during my graduate studies in the Department of Entomology.

I would like to thank Julia Guzova for her technical help during the dose response experiments, Dr. Jan Veenstra for his invaluable comments and suggestions and Dr. Teresa Martinez for her friendship during my stay in Arizona.

I thank my parents for their help and understanding. I wish to thank Mary Ellen McAsey for helping me realize my goal. Her constant support throughout my graduate career will always be remembered. Lastly, I would like to thank my son, Craig for his understanding during my graduate work.

TABLE OF CONTENTS

TABLE OF CONTENTS-*Continued*

TABLE OF CONTENTS-*Continued*

TABLE OF CONTENTS-*Continued*

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

The post blood meal diuresis in the mosquito may be regulated by neuroendocrine factors. Two families of neuropeptides, the diuresins and the leucokinins stimulate fluid secretion in Malpighian tubules of several insects. Recently, several new peptides have been isolated and synthesized from both families of neuropeptides. A putative diuresin-like diuretic factor has been purified and sequenced from *Culex salinarius*. Several leucokinins have been isolated from two species of mosquitoes, the culekinin depolarizing peptides (CDP-I, II and III) isolated from *C. salinarius* and *Aedes* leucokinin peptides (ALP-I, II and III) isolated from *Aedes aegypti*. The three major objectives of this work were: 1) to examine the effect of 5-hydroxytryptamine (5-HT), the mosquito diuresin and the mosquito leucokinins on urine production in vivo in the mosquito *A. aegypti*, 2) to inhibit the biological effects of ALP-I and *C. salinarius* diuresin with antibodies raised against these peptides, 3) to determine if biologically active peptides increased Malpighian tubule intracellular second messengers 3',5'-cyclic adenosine monophosphate (cAMP) and or inositol 1,4,5-trisphosphate (IP_3).

This study has demonstrated that putative diuretic factors from two families of insect neuropeptides and 5-HT influence Malpighian tubule function. The mosquito diuresin, leucokinins and 5-HT stimulated total urine production in a dose dependent manner. A significant inhibition of peptide-stimulated urine production occurred following immunization with either ALP-I or *C. salinarius* diuresin antibodies. The *C. salinarius* diuresin significantly ($p < 0.05$) increased intracellular cAMP concentrations in *A. aegypti* tubules. Stimulation of *A. aegypti* tubules with either CDP-II or 5-HT resulted in significant increases in both intracellular cAMP and IP_3 concentrations. All of the mosquito leucokinins, with the exception of CDP-I, significantly increased intracellular IP_3 in tubules. Data presented here suggest that the mosquito leucokinins may function on the Malpighian tubules of *A. aegypti* by increasing intracellular Ca^{2+} through the release of IP_3 sensitive intracellular Ca^{2+} stores.

1. INTRODUCTION

Insects dwell in diverse habitats, yet maintain the ionic composition and osmotic pressure of their blood (hemolymph) within a relatively narrow range (Stobbart and Shaw, 1974; Chapman, 1982). Homeostasis of the hemolymph within a narrow range is critical as most terrestrial insects, including the mosquito, have a large surface to volume ratio and must compensate for large evaporative water losses by conserving water (Phillips, 1981). Maintenance of hemolymph integrity is a major function of the excretory system of insects and is accomplished with the production of an isosmotic primary urine by the Malpighian tubules followed by a selective reabsorption of solutes, nutrients and water in the hindgut (Phillips, 1983; Phillips et al., 1986).

Malpighian tubule anatomy and physiology

This study is concerned with putative endocrine factors which regulate the Malpighian tubules in the yellow fever mosquito, *Aedes aegypti*. The focus here will be primarily on the biology of the mosquito tubules. The tubule structure of *A. aegypti* has been described only for the adult but they are structurally similar to larval tubules of the saline and brackish water mosquitoes (Mathew and Rai, 1976; Bradley et al., 1990). Ultrastructural studies of larval Malpighian tubules of *Aedes taeniorhynchus* provide a majority of the descriptions concerning mosquito tubule structure (Bradley et al., 1982). In the adult mosquito the Malpighian tubules consist of five blind-ended tubules that are surrounded by the hemolymph within the abdomen. The open end of the tubules join the alimentary canal just posterior to the midgut. The Malpighian tubule is a simple epithelium consisting of two cell types; large polarized principal or primary cells and smaller, star shaped, stellate cells. The principal cell basolateral membrane that faces the hemolymph is greatly infolded, forming multiple cytoplasmic compartments that contain mitochondria. The apical or luminal, membrane of the principal cell possesses two types of microvilli. The larger, more abundant microvilli have associated mitochondria; smaller microvilli do not contain mitochondria but can contain extensions of the rough endoplasmic reticulum. The large microvilli contain a microfilament network that may function to position the mitochondria within these structures. Microvillar volume and surface area, and mitochondrial volume within the microvilli, have been positively correlated with ability of the tubule to transport solute and fluid (Bradley and Snyder, 1989). Junctions between

principal cells near the apical membrane are continuous septate junctions that surround the cell and separate the microvillate regions from the basal infoldings. The stellate cell is essentially a thin band of cytoplasm between the tubule lumen and the hemolymph. The stellate cells are considerably smaller than the principal cells and have mitochondria associated with the basal lateral membrane of the cell. The stellate cell has an apical membrane consisting of shallow microvilli containing microfilaments without associated mitochondria (Bradley, 1985). At the junctions between stellate cells and principal cells, septate junctions extend over nearly two-thirds of the interface. The role of the stellate cell in Malpighian tubule function remains unclear, however recent studies have suggested a possible role in water transport through the actions of specialized water channels (Dow et al., 1995). Principal cells are the primary site of solute flow across the tubules (Sawyer and Beyenbach, 1985). It is this solute flow, across the tubule, that is responsible for fluid movement into the tubule lumen forming the primary urine.

The production of the primary urine by the Malpighian tubules is driven by the active transport of K^+ (or Na^+ in blood feeding insects) and Cl^- from the hemolymph (Phillips, 1981). The movement of water into the tubule lumen from the hemolymph is mediated through local osmotic processes, although the exact mechanism remains unclear (Hill, 1980; Maddrell, 1980). The process of osmotically driven fluid transport across the epithelium is probably via the standing osmotic gradient model developed by Diamond and Bossert (1968). In this model the extensive apical and basolateral infoldings of the epithelium function as sites of local solute-water coupling.

In addition to solute-water transport, insect Malpighian tubules are capable of transporting large organic molecules such as sucrose and inulin. It appears that the transport pathway for large organic molecules is different from that of the solute-water pathway across the tubule epithelium. It has been suggested that water flow is driven by solute-water coupling and occurs across the apical and basolateral membranes while large organic molecules move between cells across the septate junctional spaces (Bradley, 1985).

In most insect tubules K^+ and associated counterions (usually Cl^-) create the osmotic gradient that draws water across the epithelium. Hemolymph K^+ enters the Malpighian tubule cell by passive processes through channels in the basolateral membrane and through secondary active transport via the electroneutral $Na^+/K^+/2Cl^-$ cotransporter (Bradley, 1987a; Nicolson, 1993). The transepithelial movement of cations into the lumen of the tubules is against an impressive electrochemical gradient as indicated by a lumen positive transepithelial voltage. The apical membrane (lumen side) of the principal cell in

the Malpighian tubule develops high transmembrane electrical potentials that are ATP dependent, implying that the apical membrane plays a major role in the active transport of cations into the tubule lumen (Gupta et al., 1976; Isaacson et al., 1989; Pannabecker et al., 1992; Leyssens et al., 1993).

Evidence supports the presence of a basolateral Cl^- channel in the tubules. Malpighian tubules exposed to the stilbene derivative SITS, that blocks anion transport, significantly decreased tubule fluid secretion rates in *A. aegypti* , suggesting the presence of anion channels (Hegarty et al., 1991). The only evidence for an apical ion channel concerns a potential Cl^- channel in *A. aegypti* , that is based on data acquired using patch clamp methods (Wright and Beyenbach, 1987). In most insects Cl^- channels at the apical membrane would seem reasonable as the electrical potential across this membrane favors the passive movement of Cl^-. These data are difficult to interpret because of the proposed paracellular or shunt Cl^- conductance in *A. aegypti* and because the evidence for tubule chloride channels is not complete (Pannabecker et al., 1993).

Preliminary evidence suggests that various transport systems are present in the basolateral membrane. Indirect evidence supports the presence of K^+ channels at the basolateral membrane. Barium, which has been shown to block K^+ channels, changes tubule electrophysiology in *Onymacris plana* and *Formica ployctena* (Nicolson and Isaacson, 1987; Nicolson and Isaacson, 1990; Weltens et al., 1992).

Many insects are insensitive to ouabain, that blocks the Na^+/K^+ ATPase (Wenning et al., 1991). Ouabain increased fluid secretion in unstimulated tubules in *Rhodnius prolixus* (Maddrell and Overton, 1988). It was suggested that ouabain inhibited the basolateral Na^+/K^+ ATPase and blocked the transport of Na^+ out of the cell at the basolateral membrane. Increases in intracellular Na^+ could result in increased cation transport across the tubule setting up enhanced fluid secretion. Evidence for the presence of the Na^+/K^+ ATPase has been presented for some insect groups including *Drosophila melanogaster* and *Locusta migratoria* based on changes in tubule electrophysiology following ouabain treatment (Wessing et al., 1986; Nicolson, 1993). The inhibition of fluid secretion by ouabain suggests the presence of a Na^+/K^+ ATPase pump (Clements, 1992; Kerkhove, 1994). Studies using vanadate, a P-type ATPase blocker similar to Na^+/K^+ ATPase also demonstrated inhibited fluid secretion in the isolated tubules of *F. ployctena* (Dijkstra et al., 1994).

An analog of cAMP, dibutyryl adenosine 3',5',-cyclic monophosphate (DB cAMP) lowered the fractional resistance of the basolateral membrane of *A. aegypti* tubules and

increased the basolateral Na^+ conductance, suggesting that cAMP selectively activates certain ion transporters (Hegarty et al., 1991). Experiments with *A. aegypti* tubules using bumetanide, a $Na^+/K^+/2Cl^-$ cotransport inhibitor, determined that cAMP may affect this transporter. Hegarty et al.(1991) demonstrated that increases in *A. aegypti* tubule fluid secretion that were stimulated by DB-cAMP could be blocked by bumetanide. Tubules treated with bumetanide also exhibited a reduction in tubule fluid secretion in *R. prolixus*, *L. migratoria*, *Glossina morsitans* and *D. melanogaster*, (Wessing et al., 1986; Nicolson, 1993). Intracellular Na^+ increased in tubules of *R. prolixus* that were stimulated with 5-hydroxytryptamine (5-HT) suggesting that this basolateral cotransporter was activated (Gupta et al., 1976). These results were explained because of the inherently low Na^+ permeability of the basolateral membrane in *R. prolixus*, tubules, implying that the cotransporter would be an important route for Na^+ to enter the cell.

Within the last few of years, research regarding the transport mechanisms of insect Malpighian tubules has undergone some exciting changes. Early studies that examined epithelial transport in insects led to the rejection of the role of the Na^+/K^+ ATPase as generator of the primary electrochemical gradient required for transport mechanisms (Keynes, 1969). Several investigators have subsequently reported that insect ion-transporting epithelia contain a unique alkali metal ion pump, the vacuolar-type proton ATPase (V-ATPase) (Harvey et al., 1983; Schweikl et al., 1989; Wieczorek et al., 1989; Bertram et al., 1991; Klein, 1992). These data explain, to some degree, the insensitivity of the insect epithelia to ouabain, because many insect epithelia do not appear to possess an active vertebrate Na^+/K^+-ATPase.

The discovery of the V-ATPase has also altered the model of the transport of solute and water in insect epithelia, including Malpighian tubules. The current model for the insect Malpighian tubule suggests that the actions of the apical V-ATPase establish the driving proton gradient for transport of ions and water (Maddrell and O'Donnel, 1992). Although the actions of the V-ATPase are primary to solute and water movement in the tubules, it is the combined actions of the V-ATPase and the antiporter complex that underlies the transport mechanisms of these epithelia. The major difference in this model compared to the previous model for Malpighian tubule transport is the replacement of the common cation pump at the apical membrane (transporting N^+, K^+ or both) with the V-ATPase and cation/H^+ antiporter(s) complex. This model and the previous model, which did not incorporate the V-ATPase, does not have a functioning Na^+/K^+ ATPase located on the basolateral membrane (Maddrell and O'Donnell, 1992). The Na^+/K^+ ATPase is critical

in maintaining the intracellular homeostatic concentrations of ions in vertebrates. The lack of a Na^+/K^+ ATPase is rare in cellular physiology. How, then, do insect epithelia fight Donnan forces and maintain a functional internal solute concentration of low Na^+ and high K^+?

Because of Donnan forces, Na^+ tends to enter the epithelial cell at the basolateral side via Na^+ channels and the $Na^+/K^+/2Cl^-$ cotransporter. Sodium moves into the cell because the cell exhibits a fixed net negative charge due primarily to the net negative charge of cellular proteins (Alberts et al., 1989). The V-ATPase functions by actively transporting protons out of the cell into the lumen of the tubule. The protons in the lumen are then recycled back into the cell by the apical Na^+/H^+ antiporter. The net affect of the transporting action of this complex is the removal of Na^+ from the cell and the recycling of protons from the cell to the lumen and back in to the cell via the cation/H^+ antiporter (Harvey, 1992).

Evidence that the V-ATPase complex is located on the apical membrane of tubules of insects in association with the cation/H^+ antiporter(s) include the binding of a V-ATPase antibody to apical membrane preparations from Malpighian tubules in *Manduca sexta* (Russell et al., 1992; Klein, 1992). Studies using amiloride, which blocks Na^+/proton exchangers, inhibited fluid transport by Malpighian tubules in *D. melanogaster* and *R. prolixus* (Bertram, 1989; Maddrell and O'Donnell, 1992). V-ATPase inhibitors such as bafilomycin A_1 (Bertram et al., 1991), N-ethylmaleimide (NEM) (Weltens et al, 1992), and 4-chloro-7-nitrobenzo-2-oxa-1,3-diazol (NBDC1) (Maddrell and O'Donnell, 1992) inhibit fluid secretion in Malpighian tubules.

In most terrestrial insects, K^+ is the major transport ion and, according to this model, fluid transport by the Malpighian tubules is mediated by a K^+/H^+ antiporter functioning in tandem with the V-ATPase at the apical membrane. This condition makes sense in that most terrestrial insects are phytophagous and have diets rich in K^+. In blood feeding insects the situation is different. During non-blood feeding periods the major transporting ion is K^+, as is the situation in most insects. Following the blood meal, Na^+ functions as the major transporting cation (Williams and Beyenbach, 1983). Sodium conductance increases at the basolateral membrane under the action of diuretic peptides (Petzel, et al., 1986). As an example, following the blood meal, *A. aegypti* females can produce rapid fluid movement, rich in both Na^+ and Cl^- (Williams and Beyenbach, 1983). Maddrell and O'Donnell (1992) suggest several mechanisms in which the V-ATPase/cation antiporter system can accommodate these demands. This can be accomplished by the

presence of a common K^+-Na^+/H^+ antiporter which would have a higher affinity for Na^+. An alternate mechanism would involve the presence of two types of antiporters, one for transporting Na^+ and another for transporting K^+ with the Na^+/H^+ antiporter present in greater numbers or functioning at relatively higher efficiencies. Based on initial experiments, Maddrell and O'Donnell (1992), concluded that a common K^+-Na^+/H^+ antiporter system is probably present in the Malpighian tubules.

In the tubule lumen, water and solutes form the primary urine which eventually passes into the hindgut. In some insects, the primary urine can move forward into the midgut where reabsorption can occur Dow (1981), or as is generally the case, into the posterior hindgut to the rectum. To function in a homeostatic manner the excretory system must not only accomplish secretion but must also function in reabsorption to reclaim important electrolytes, nutrients and water from the urine. The reabsorption of important components from the urine occurs at either the proximal Malpighian tubule Maddrell and Phillips (1975), or the rectum in the posterior hindgut (Phillips, 1983). The selective reabsorption by the hindgut is an active process and can create a final urine which is hypo or hyperosmotic to the hemolymph, depending on rates of water and ion absorption (Spring and Clark, 1990).

Insect diuresis

Most insects face the challenge of conserving water throughout their life cycle to prevent desiccation and because of this, the rapid excretion of urine (diuresis) is not common. However, at selected times in the life cycle of some insects, a diuresis is necessary.

Diuresis following eclosion. Many insects undergo a diuresis following eclosion in order to improve flight ability by reducing larger hemolymph loads carried during the early stages of life and to remove metabolic wastes which have accumulated during insect development (DeGuire and Fraenkel, 1973; Gillett, 1982; Gillett, 1983; Clements, 1992).

Diuresis as a clearance mechanism. The desert beetle *O. plana* undergoes a process, similar to diuresis, in which secretion by the Malpighian tubules is increased but total water loss is greatly reduced (Nicolson and Hanrahan, 1986). In this case, increased secretion from the tubules is countered by reabsorption at the hindgut, resulting in a conservation of solutes and water from the urine. The primary physiological actions of this

process are to clear toxic components from the insect while conserving water and important solutes (Maddrell, 1980; Nicolson, 1991).

Diuresis following the blood meal. Haematophagous insects undergo a diuresis to compensate for the physiological challenges encountered as a result of the blood meal. Blood feeding insects periodically ingest large blood meals. The adult female yellow fever mosquito, *A. aegypti* can ingest a meal nearly two fold larger than its total body mass, while the blood feeding insect, *R. prolixus* can ingest more than 10 times its body mass (Maddrell, 1963; Williams and Beyenbach, 1983). It is apparent that blood feeding insects must possess an excretory system that can mediate a rapid and sustained diuresis for the removal of excess water and solutes from the hemolymph.

The diuresis after the blood meal accomplishes three functions critical to the survival of the mosquito: 1) a reduction in body weight to optimize maneuverability, 2) a concentration of the nutrient rich blood cells in the midgut and 3) a restoration of normal hemolymph volume and ionic composition (Bradley, 1985).

As a result of the blood meal, the female mosquito is presented with an increased Na^+ and Cl^- load from a fluid with a greatly reduced osmolality, relative to the hemolymph (Williams and Beyenbach, 1983). A major function of the diuresis is to remove excess Na^+ and water that is absorbed across the midgut from the blood meal. This occurs primarily during the peak phase of diuresis, when the urine production rate is highest and Na^+ and Cl^- are the predominant ions in the urine. During this period of high flow rates, Na^+ and Cl^- support the movement of a majority of the water lost during the diuresis. After the initial phase of diuresis, urine K^+ concentrations increase, Na^+ concentrations decrease and the urine production rate is reduced. In general, higher urine Na^+ concentrations have been correlated with higher urine production rates and higher urine osmolality. High urine K^+ concentrations has been correlated with lower urine production rates and with lower urine osmolality (Clements, 1992).

The post blood meal diuresis is a relatively efficient process. Nearly 42% of the plasma volume, 44% of the Na^+ and 58% of the Cl^- from the plasma load are excreted during diuresis in the mosquito (Williams and Beyenbach, 1983). Greater than 100% of the K^+ plasma load is excreted during the diuresis indicating that some of the K^+ excreted is probably contributed from the breakdown of the erythrocytes within the midgut.

Endocrine regulation of tubule function

Diuretic factors that stimulate diuretic activity have been found throughout the insect nervous system including the brain, ventral nerve ganglia, corpora cardiaca (CC), and corpora allata (CA) (Maddrell, 1963; Wall and Ralph, 1964; Berridge, 1966; Mills, 1967; Nicolson, 1976; Proux et al., 1982; Morgan and Mordue, 1984; Coast, 1988). Stimulation of secretion by Malpighian tubules, using crude extracts from nervous tissues of blood feeding insects, initially suggested that the neuroendocrine regulation of tubule function maybe important. Studies by Gee (1975, 1976), using *G. morsitans* demonstrated that neural extracts could increase secretion in isolated tubules. Experiments using nervous tissue extracts from *R. prolixus* demonstrated similar results in isolated tubules (Hughes, 1979). These experiments indicated that Na^+ was the major cation transported during post blood meal diuresis and revealed that the second messenger 3',5'-cyclic adenosine monophosphate (cAMP) may mediate the actions of these putative neuroendocrine factors at the tubule (Gee, 1976). These data strongly suggested that neuroendocrine factors regulated diuresis.

Three putative diuretic factors isolated from mosquito head extracts

Experiments using crude saline extracts from the heads of adult female mosquitoes suggested that diuresis is regulated by neuroendocrine factors. Treatment of isolated tubules with crude neural extracts altered tubule transepithelial voltage and increased tubule fluid secretion rates (Nijhout and Carrow, 1978; Phillips, 1983; Williams and Beyenbach, 1983). Three biologically active factors, obtained by high pressure liquid chromatography (HPLC) fractionation of head extracts of female *A. aegypti*, were biologically active on the mosquito Malpighian tubule (Petzel, 1985; Petzel, et al., 1986; Wheelock, et al., 1988). These factors were evaluated for their ability to alter tubule electrophysiology in a reversible manner, to increase tubule fluid secretion, and to increase urine production (Table 1.1) (Petzel, 1985; Petzel et al., 1986; Wheelock et al., 1988).

Factor I caused a depolarization of tubule transepithelial voltage, and increased urine production rate, but did not increase isolated tubule fluid secretion or change intracellular cAMP concentrations. Factors II and III altered tubule electrophysiology, increased both urine production rate and tubule fluid secretion (Petzel et al., 1986; Wheelock et al., 1988). Factor II caused a depolarization in transepithelial voltage,

Table 1.1. Effects of HPLC factors, from head extracts, on Malpighian physiology in *A. aegypti*.

HPLC Factor	Transepithelial voltage change	Isolated tubule fluid secretion	Urine production	Tubule intracellular cAMP
Factor I	depolarize	none	increase	none
Factor II	depolarize	increase	increase	increase
Factor III	depol/hyperpol*	increase	increase	increase

*depol/hyperpol. = transient depolarization followed by a hyperpolarization

whereas factor III stimulated a transient depolarization followed by a hyperpolarization in transepithelial voltage. Both factors II and III stimulated tubule intracellular cAMP concentrations; factor III was more potent than factor II (Petzel et al., 1987). Both factors increased urine NaCl and decreased urine KCl secretion in the mosquito, suggesting a possible role in regulating urine production (Petzel, 1985). Petzel and his coworkers (1985, 1987), concluded that factor III was an important diuretic factor in the mosquito. Components of factor III function on the tubules in a cAMP dependent manner to stimulate NaCl secretion. Factor III was thus termed "mosquito natiuretic factor" (Petzel, 1985; Petzel et al., 1987; Wheelock et al., 1988; Thompson et al., 1995).

The search for hormones regulating tubule fluid secretion in the mosquito and other insects has involved the investigation of different groups of putative diuretic hormones. These putative hormones can be classified into several chemical families. A discussion of different groups of putative diuretic agonists will be helpful in understanding the development of research into putative diuretic factors in mosquitoes.

Arginine-vasopressin-like peptides

The first insect diuretic hormone isolated, sequenced and synthesized was from the migratory locust, *L. migratoria*. Crude extracts were made from ganglia, separated using HPLC and active factors were identified (Proux et al., 1987a, b). It was eventually established that the diuretic hormone was a dimer (Proux et al., 1987a, b; Schooley et al., 1987). This peptide was found to be homologous to the arginine-vasopressin peptide family and was termed the arginine-vasopressin-like peptide (AVP-like peptide) (Proux et al., 1987b; Schooley et al., 1987). One of the actions of arginine-vasopressin in vertebrates is at the collecting tubules in the kidney and functions in regulating water

reabsorption (Robertson et al., 1977) by influencing a specialized water channel in the kidney (DiGiovanni et al., 1994). Identification of vasopressin-like immunoreactive tissue in the neurons of *L. migratoria* was corroborated in studies using antibodies raised against lysine vasopressin (Thompson et al., 1991; Petzel and Samuelson, 1992; Thompson et al., 1995). Other studies correlated AVP-like peptide immunoreactivity in the hemolymph of locusts with changes in the rate of dye excretion from locusts, which was utilized as an indicator of diuretic activity (Picquot and Proux, 1988). Since AVP-like peptide immunoreactivity in the hemolymph was not correlated with all changes that occurred in the urine production rate questions were raised about the significance of this peptide in regulating tubule physiology. Initial studies suggested that AVP-like peptide stimulated increases in tubule cAMP concentrations Picquot and Proux (1988), however, Coast et al. (1993) reported that the AVP-like peptide did not stimulate fluid secretion or intracellular cAMP concentrations in *L. migratoria* tubules. These data question the importance of the AVP-like peptide in regulating tubule function. The identification of an AVP-like peptide was a significant achievement, however, as recent immunohistochemical studies have determined that this peptide is found only in insects closely related to *L. migratoria* (Evans and Cournil, 1990; Davis and Hildebrand, 1992). Until representatives of this family of peptides having diuretic activity are isolated from other insect groups their role in regulating Malpighian tubule function will remain uncertain.

The eicosanoids

The eicosanoids, which are oxygenated metabolites of arachidonic acid, have been implicated in Malpighian tubule regulation. Indirect evidence based on studies utilizing inhibitors targeted at different eicosanoid biosynthesis pathways suggest that the eicosanoids may function in regulating basal tubule physiology. Experiments in which isolated Malpighian tubules of *A. aegypti* were treated with the phospholipase A_2 inhibitor, 5, 8, 11, 14-eicosatetraynoic acid (ETYA), which blocks the biosynthesis of all the eicosanoids products, significantly reduced baseline tubule fluid secretion (Petzel and Samuelson, 1992). To investigate which group of eicosanoids were involved, inhibitors were applied to tubules to block either the cyclooxygenase or the 5-lipoxygenase biosynthesis pathways. These experiments indicated that products from the eicosanoid cyclooxygenase pathway were involved in regulating tubule function (Petzel and Samuelson, 1992). The prostaglandins are an important product of the cyclooxygenase pathway and have been shown to have a role in vertebrate renal function (Bonvalet et al.,

1987). Petzel and Samuelson (1992), suggest that the prostaglandins may regulate tubule function. Immunohistochemical identification of prostaglandin PGE_2 in the Malpighian tubules of *A. aegypti* supports the suggestion that the eicosanoids are involved in tubule function (Petzel, et al., 1993).

5-hydroxytryptamine

The role of 5-hydroxytryptamine (5-HT) as a putative endocrine factor has been investigated in several insect groups. Physiological studies suggest that separate 5-HT receptors exist in the several insects including the CC of *Periplaneta americana* (Gole et al., 1987), the salivary gland in *M. sexta* (House and Ginsborg, 1985), forgut of *Schistocerca gregaria* (Banner et al., 1987) and mandibular muscles of *Gryllus domestica* (Baines and Downer, 1991). Recent studies by Hiripi and Downer (1993), provide convincing evidence for 5-HT receptors in *Locusta* brain tissue. The in vitro treatment of isolated Malpighian tubules with 5-HT results in increased tubule fluid secretion has formed the bases for speculation that this biogenic amine may be active in several insect groups. In non-blood feeding insects 5-HT increased tubule fluid secretion in the following groups: *Carausius morosus* (Maddrell et al., 1971), *L. migratoria* (Morgan and Mordue, 1984), *Papilio demodocus* (Nicolson and Millar, 1983) and *Calliphora vicina* (Schwartz and Reynolds, 1979). Data provided from these studies suggests that 5-HT may have a role in regulating fluid secretion in the tubules. It is, however, difficult to interpret the physiological relevance of these studies since in some cases, the 5-HT concentrations used were exceptionally high.

The role of 5-HT as a diuretic hormone has been established in only two insect, *R. prolixus* and larval *A. aegypti*. The blood feeding bug *R. prolixus* takes a large blood meal and must compensate for the increased Na^+ and fluid load by rapidly eliminating these components via a diuresis (Ramsay, 1952; Maddrell, 1963). Previous studies have shown that 5-HT stimulated tubule fluid secretion in the tubules of *R. prolixus* at relatively low concentrations (Maddrell et al., 1969; Maddrell et al., 1971). Concentrations of 5-HT in the hemolymph of *R. prolixus* increase during the blood meal to levels which have been shown to increase tubule fluid secretion in vitro (Lange et al., 1989). 5-HT has also been identified immunohistochemically in the nervous tissues Lange et al. (1988), and a specific 5-HT receptor has been identified in the tubules of *R. prolixus* (Maddrell et al., 1991). Although, Malpighian tubule function in *R. prolixus* is complicated by the existence of a

separate diuretic peptide Maddrell et al. (1991), these data provide convincing evidence that 5-HT functions as a diuretic hormone in *R. prolixus*.

5-HT receptors have been identified in some insect species including *C. morosus* Maddrell (1971), and most notably in *D. melanogaster* (Saudou et al., 1992). Berridge (1981), has isolated two receptors in *C. vicina* salivary gland and has suggested a mechanism for this biogenic amine in insects. One receptor activated the cAMP cellular pathway while the other functioned via the Ca^{2+}/phosphatidylinositol (IP_3) pathway (Berridge, 1981; Berridge and Heslop, 1981; Berridge, 1983). 5-HT has also been shown to stimulate Malpighian tubules in *L. migratoria* via a Ca^{2+} dependent pathway (Morgan and Mordue, 1984; Morgan and Mordue, 1985a). In *R. prolixus*, 5-HT significantly stimulates intracellular cAMP in Malpighian tubules Barrett and Orchard (1990), and may influence the basolateral $Na^+/K^+/2Cl^-$ cotransporter (O'Donnell and Maddrell, 1984).

In the mosquito, *A. aegypti*, 5-HT immunoreactive cells have been identified in the neurons surrounding the salivary gland of the adult female (Novak et al., 1995). Veenstra (1988), examined various biogenic amines and reported that only 5-HT increased tubule fluid secretion and altered tubule transepithelial voltage. In larval *A. aegypti*, 5-HT acts on the tubules and is important in regulating ion and water balance. Hemolymph titers of 5-HT rise as the insect is placed in increasingly saline conditions (Clark, 1994). 5-HT stimulates increases in larval tubule fluid secretion containing NaCl and KCl (Clark and Bradley, 1996). Diuresis, in aquatic larvae, functions in the removal of water, compensating for an inwardly directed osmotic gradient (Bradley, 1987b). This diuresis is brought about by a different stimulus as compared to a post blood meal diuresis. Clark and Bradley (1997), present a pharmacological approach to characterizing the 5-HT receptor at the Malpighian tubules of larval *A. aegypti*. The evidence suggests that *A. aegypti* tubules possess a unique insect 5-HT receptor. Based on the 5-HT receptor affinity for the antagonist (+)-butaclamol, it was found that the *A. aegypti* receptor could be pharmacologically similar to the 5-HT receptor found in the CC of *P. americana* (Clark and Bradley, 1997). 5-HT has been shown to increase Malpighian tubule fluid secretion in many insect groups, 5-HT immunoreactivity has been identified in insect neural tissues and receptors have been characterized in some insect groups. The importance of 5-HT in regulating tubule function in mosquitoes and other insects will therefore remain a important question in insect physiology. Studies considering the 5-HT receptor, mechanism(s) of

action and immunohistochemical identification will be needed to further explore the function of 5-HT in tubule physiology.

The two remaining families of putative diuretic factors that stimulate fluid secretion in the Malpighian tubules of mosquitoes as well as other insects are the leucokinins and the corticotropin-releasing factor-like diuretic hormones (CRF-like DH), also known as the diuresins. Both peptide families are more widely distributed throughout the insects as compared to the other factors discussed above.

The leucokinins

The leucokinins are a group of myotropic neuropeptides, that may regulate diuresis in insects. The leucokinins are octapeptides that share a pentapeptide amide C-terminal region characterized by the amino acid sequence Phe-X1-X2-Trp-Gly-NH_2, where X1 is Asn, His, Phe or Tyr and X2 is Ser, Pro or Ala (Holman et al., 1990). The conserved C-terminal core region may be important for biological activity Nachman et al. (1995), and the N-terminal sequence may influence biological potency (Nachman et al., 1992). Originally isolated from the head extracts of *Leucophaea maderae*, active factors were evaluated based on their ability to stimulate hindgut contractions (Holman et al., 1986; Holman et al., 1986; Holman et al., 1987a; Holman et al., 1987b). The leucokinins have been shown to stimulate fluid secretion in isolated Malpighian tubules in *A. aegypti* and *Acheta domesticus* (Hayes et al., 1989; Coast et al., 1990). Leucokinin-like peptides have been identified and sequenced from a number of insect species using bioassays for either hindgut myotropic activity or increased tubule fluid secretion. To date, the following insect groups have had leucokinin-like peptides identified and sequenced: eight peptides from *L. Maderae* (Holman et al., 1986a, b; 1987a, b), five peptides from *A. domesticus* (Holman et al., 1990), a single peptide from *L. migratoria* (Schoofs et al. (1992), and three peptides from *Helcoverpa zea* (Blackburn et al., 1995). In the mosquito, three leucokinin-like peptides have been isolated from whole body extracts of *Culex salinarius*, termed the culekinin depolarizing peptides (CDP) (Hayes et al., 1994). Three leucokinin-like peptides have been isolated from headless *A. aegypti* and termed *A. aegypti* leucokinin peptides (ALP) (Veenstra, 1994).

Leucokinin immunoreactive neurosecretory cells have been identified in many insect groups. Antibodies raised against leucokinin I from *L. maderae* was used to identify immunoreactive cells in the abdominal ganglia of *L. migratoria*, the cockroach *L. maderae*, the lepidopteran larvae *Agrotis segetum* and in the flies; *C. vicina*, *D. melanogaster*,

Phormia terraenovae and larvae of *Phalacrocera replicata* (Cantera and Nassel, 1992; Nassel et al., 1992; Cantera et al., 1992; Thompson et al., 1995). Antibodies raised against leucokinin IV of *L. maderae* was used to identify immunoreactive neurosecretory cells in nervous tissues of *M. sexta*, *Nauphoeta cinerea*, *A. domesticus*, *Apis mellifera*, and *Schistocerca americana* (Chen et al., 1994a, b). In the mosquitoes, immunohistochemical studies using an antiserum against achetakinin-I that shares the pentapeptide amide C-terminal amino acid sequence with CDP-2, have localized immunoreactive neurons in *C. salinarius* (Clottens et al., 1993). In the adult and larvae of *A. aegypti*, leucokinin IV immunoreactive neurosecretory cells have been identified in both the brain and abdominal ganglia (Chen et al., 1994a). A cDNA has been isolated from *A. aegypti* and found encode for a preproleucokinin, indicating that the three *A. aegypti* leucokinins are represented by a single gene (Veenstra et al., in press).

Chung et al. (1995), have presented indirect evidence for leucokinin specific receptors. In these experiments, a radiolabeled analog to achetakinin II bound rapidly and reversibly to plasma membrane preparations from the Malpighian tubules of *A. domesticus*. In mosquitoes, at least two leucokinin receptors have been tentatively identified in *A. aegypti* (Hayes, personal communication).

Hayes et al. (1989), reported that Leucokinin VIII from *L. maderae* depolarized transepithelial voltage of *A. aegypti* Malpighian tubules and increased tubule fluid secretion. This effect was diminished if the Cl^- concentration in the tubule bathing media was reduced, indicating that the leucokinins function in a Cl^- dependent manner (Hayes et al., 1989). Leucokinins increased the secretion of NaCl, KCl and water by increasing Cl^- conductance across the tubule in an undetermined paracellular pathway. The major cations, Na^+ and K^+ cross the tubule through active transport processes via the principal cells in *A. aegypti* (Williams and Beyenbach, 1984). The major counter ion, Cl^- moves passively across the tubule epithelium, driven by electrochemical potentials established by total solute flow (Williams and Beyenbach, 1984).

Because cation transport is an active mechanism, studies investigating tubule electrophysiology have utilized dinitrophenol (DNP), uncouples oxidation phosphorylation (depletes cellular ATP), and effectively blocks active cation transport (Issacson et al., 1989; Leyssens et al., 1991; Pannabecker et al., 1992). If active transport mechanisms are blocked at the principal cells of the tubule, then transepithelial resistance would be substantially increased. This is precisely the effect found in studies that applied DNP to isolated tubules of *A. aegypti* (Leyssens et al., 1991; Pannabecker et al., 1992). Tubule

conductance across the tubule was inhibited by DNP because cation transport is primarily responsible for tubule conductance. In the presence of DNP transepithelial resistance thus represents the resistance across an alternate pathway into the tubule lumen. The alternate route into the lumen of the tubules or paracellular route represents either a pathway between the cells (intercellular pathway) or across the minor cell type of the tubule, the stellate cells (Pannabecker et al., 1993). In experiments with *A. aegypti* in which DNP treated tubules were exposed to leucokinin VIII, the paracellular resistance decreased by more than 7 fold with a concomitant increase in Cl^- conductance (Pannabecker et al., 1993). In these studies changes in Cl^- conductance was assumed not to occur across the principal cells, because of the actions of DNP, but rather involved a paracellular pathway. These data have shown that the leucokinins some how influence the passive movement of Cl^- across the tubule via a paracellular pathway that enhances fluid movement into the lumen.

Are the leucokinins biologically active? The leucokinins may influence tubule physiology in mosquitoes as well as other insects. Chung et al. (1994), have measured achetakinin concentrations in the hemolymph of *A. domesticus* and shown that changes in leucokinin concentrations correlated with changes in feeding status. These studies suggest that, leucokinins, released into the hemolymph, influence tubule physiology. Leucokinins from *L. maderae* stimulated fluid secretion in isolated tubules and depolarized Malpighian tubule transepithelial voltage in a chloride-dependent manner in *A. aegypti* (Hayes et al., 1989; Pannabecker et al., 1993; Hayes et al., 1994). The mosquito leucokinins, the CDPs and ALPs, depolarized tubule transepithelial voltage, suggesting biological activity (Hayes et al., 1994; Veenstra, in press).

When injected into mosquitoes, factor II, from head extracts of *A. aegypti*, increased urine production (Wheelock et al., 1988). Factor II also increased fluid secretion in a chloride-dependent manner and depolarized tubule transepithelial voltage in isolated tubules. These results suggested that factor II may be a leucokinin-like peptide factor (Hayes et al., 1989). These data suggest a possible role for the leucokinins in regulating Malpighian tubule physiology in the mosquito.

Leucokinins and cellular signal transduction. The intracellular signaling mechanism for the leucokinins is not well understood but cAMP independent mechanisms may be important. There are examples of insect endocrine factors that function in a cAMP independent manner. In *M. sexta*, the cardioacceleratory peptides, CAP1 and CAP2, effect the myocardium through changes in intracellular IP_3 (Tublitz, 1988). Allatotropin is active at the CA through an IP_3 second messenger pathway (Reagan et al., 1992). In *L.*

migratoria, proctolin influences ovary and hind leg muscle contraction via IP_3 (Worden and O'Shea, 1986; Lange, 1988). Juvenile hormone was shown to affect male accessory gland protein synthesis in *D. melanogaster* via an IP_3 dependent signal transduction mechanism (Yamamoto et al., 1988). Eclosion hormone is active at the abdominal ganglia via IP_3 in *Bombyx mori* (Shibanaka et al., 1993). In *Lymantria dispar* ecdysiotropin is active on the testes via both cAMP and IP_3 mediated cellular mechanisms (Loeb, 1993). In locusts, two distinct diuretic factors were isolated from CC extracts; only one of which stimulated tubule fluid secretion in a cAMP dependent manner (Morgan and Mordue, 1985b). Exposure of isolated tubules to CC extracts stimulated both cAMP and IP_3 (Fogg et al., 1990). Achetakinins increased tubule fluid secretion independently of cAMP, probably through changes in cellular Ca^{2+} (Coast et al., 1990; Coast and Kay, 1994). In *D. melanogaster*, leucokinin-stimulated increases in tubule secretion were independent of both cAMP and cGMP. Increases in tubule secretion were similar to that exhibited by tubules stimulated with the Ca^{2+} mobilizing drug, thapsigargin (Davies et al., 1995). In *D. melanogaster*, leucokinin mediated changes in tubule Cl^- conductance were demonstrated to be dependent on cellular Ca^{+2} and distinct from cation transport processes (O'Donnell et al., 1996).

The mosquito leucokinins cause a depolarization in the transepithelial voltage, stimulate fluid secretion and are likely biologically active in the mosquito (Hayes et al., 1994; Veenstra, in press). In the mosquito, the physiological effects of the leucokinins, including depolarization and fluid secretion, are mediated through the hydrolysis of phosphatidylinositol leading to the production of the cellular second messenger IP_3 that is associated with transient increases in cellular Ca^{2+}.

The CRF-like diuretic peptides

The corticotropin-releasing factor diuretic hormones (CRF-like DH), also known as the diuresins, share amino acid sequence similarities to the corticotropin-releasing factor (CRF) family of peptides. These peptides are structurally similar to the vertebrate peptides: corticotropin-releasing factor, urotensin I and sauvagine (Schooley, 1993). The vertebrate CRF, originally isolated from ovine hypothalami, have since been sequenced from several other vertebrate groups (Vale et al., 1981). CRF plays a major endocrine role in vertebrates in regulating the secretion of an adrenal cortical stimulating hormone (ACTH) from the anterior pituitary (Hadley, 1988). ACTH stimulates steroid biosynthesis within the adrenal steroidogenic tissues resulting in the production and release of the glucocorticoids which are involved in carbohydrate metabolism. The CRF family of

peptides also have hypotensive/vasodilatory effects due primarily to the antidiuretic actions of sauvagine. Sauvagine, originally isolated from the skin of the frog *Phyllomedusa sauvagei*, was evaluated for bioactivity based on its antidiuretic actions on the rat (Montecucchi and Henschen, 1981). Urotensin I was originally isolated from the urophysis, analogous to the hypothalamus of bony and cartilaginous fishes (Bern et al., 1985). Urotensins have a variety of actions in vertebrates including vasopressor and vasodepressor effects (Hadley, 1988). In general, these peptides stimulate hypotensive/vasodilatory effects in mammals (Ichikawa et al., 1982). The amino acid sequence identity between the vertebrate and insect forms of these peptides is about 17% suggesting that cross reactivity may not be significant. The vertebrate forms of these peptides show no biological activity in insects (Audsley et al., 1995).

Kataoka et al. (1989), first isolated and sequenced a representative of this group from *M. sexta*. Head extracts from pharate adults were purified using HPLC and evaluated for biological activity in an in vivo bioassay for urine production in adult *Pieris rapae* and larval *M. sexta* (Kataoka et al., 1989). The diuresins are potent stimulators of Malpighian tubule fluid secretion and stimulate increases in tubule intracellular cAMP (Troetschler and Kramer, 1992; Audsley et al., 1995). The ability of these peptides to stimulate tubule fluid secretion and intracellular cAMP has been utilized to evaluate active factors in studies identifying new members of this peptide group. In insects, seven members of the CRF family of peptides have been identified and sequenced; two from *M. sexta* (Kataoka et al., 1989; Blackburn, 1991), and one each from *A. domesticus* (Kay, et al., 1991a), *L. migratoria* (Kay et al., 1991b; Lehmberg et al., 1991), *P. americana* (Kay et al., 1992), one identical peptide from *Musca domestica* and *Stomoxys calcitrans* (Clottens et al., 1994) and *Tenebrio molitor* (Furuya et al., 1995). A diuresin has been tentatively identified and sequenced from *C. salinarius* (Clottens, personal communication).

CRF-like immunoreactive cells have been identified in neurosecretory cell axon terminals in the CC of *M. sexta* and in *Eurema nicippe* with antibodies raised against the N- and C- terminal portions of the *M. sexta* diuresin (Veenstra and Hagedorn, 1991). A second *M. sexta* diuresin, Mas DP II, synthesized in the neurohemal corpora cardiaca-corpora allata (CC-CA) complex has also been identified (Blackburn, 1991). In *L. migratoria*, polyclonal antibodies were raised against the C-terminal region of the *Locusta* diuresin and used to identify immunoreactive cell bodies in the brain, CC, perivisceral organs and abdominal ganglia (Patel et al., 1994). Purified factors from the hemolymph coeluted with the synthetic *Locusta* diuresin (Patel et al., 1994). Lehmberg et al. (1993),

identified *Locusta* diuresin immunoreactivity, by means of a modified competitive enzyme-linked immunosorbent assay (ELISA), in the brain, storage lobes of the CC and hemolymph of locusts. These data suggest that the *Locusta* diuresin is present in the brain and the neurohemal sites of the CC where it is released, via Ca^{2+} dependent mechanisms, into the hemolymph (Patel et al., 1994).

Increases in both intracellular cAMP and fluid secretion occurred in adult *M. sexta* tubules following treatment with *M. domestica* diuresin in vitro (Clottens et al., 1994). Audsley et al. (1993), demonstrated that the *M. sexta* diuresin activated tubule fluid secretion in adult tubules and had an antidiuretic effect at the cryptonephric complex of larval *M. sexta*. These actions have been shown to be cAMP mediated on both the adult tubules and the cryptonephric complex in the larvae of *M. sexta* (Audsley et al., 1993). Additionally, the second M. sexta diuresin, Mas DP II, has a diuretic function. Injections of this diuresin into decapitated adult *M. sexta* increased fluid loss in a dose dependent manner (Blackburn, 1991; Blackburn and Ma, 1994). In the locust, isolated Malpighian tubules treated with the *Locusta* diuresin significantly increased tubule fluid secretion and tubule intracellular cAMP in a dose dependent manner (Lehmberg et al., 1991; Lehmberg et al., 1993). Kay et al. (1991b, 1992), demonstrated that diuresins of *Acheta* and *Periplaneta* stimulated an increase in fluid secretion and intracellular cAMP in isolated Malpighian tubules in these groups. These data suggest that the increase fluid secretion by the Malpighian tubules occurred via the actions of the cellular second messenger cAMP.

A cDNA clone has been isolated and encodes a precursor form of the *M. sexta* diuresin (Digan et al., 1992). *M. sexta* diuresin mRNA was found to be expressed in the brain, nerve cord, gut and Malpighian tubules of *M. sexta* larvae (Digan et al., 1992). The expression of this diuresin in tubule tissue, the major target organ of this peptide, could suggest a possible paracrine action of this hormone on the Malpighian tubule.

A cDNA encoding the *M. sexta* diuresin receptor has been isolated from larval Malpighian tubules of *M. sexta* and may be a G-protein coupled receptor (Reagan, 1994). COS-7 cells transfected with the *M. sexta* receptor gene exhibited a significant increase in intracellular cAMP following exposure to the diuresin. These data, along with the suggested receptor conformation, indicate that the *M. sexta* diuresin receptor is coupled to the adenylate cyclase signal transduction pathway (Reagan, 1994).

There is evidence suggesting the diuresins function by influencing the basolateral $Na^+/K^+/2Cl^-$ cotransporter. In the cryptonephric complex of larval *M. sexta*, the *M. sexta* diuresin influences the basolateral cotransporter. Audsley et al. (1993), demonstrated that

treatment of the cryptonephric complex of larval *M. sexta* with bumetanide, an inhibitor of the $Na^+/K^+/2Cl^-$ cotransporter, inhibited the antidiuretic activity of the M. sexta diuresin. Removal of Na^+ or K^+ or Cl^- also inhibited the antidiuretic actions of this peptide (Audsley et al., 1993).

Are the diuresins biologically active? Evidence for the biological significance of the diuresins is provided at several different levels. As described above, diuresins stimulate tubule secretion via cAMP mediated mechanism in several insect groups. Diuresin-like immunoreactive neurohemal cells in the nervous tissues of insects have been identified. Diuresins in the hemolymph of insects have also been indirectly measured. Additionally, diuresin receptor and diuresin precursor cDNA has been isolated from Malpighian tubules. Immunoneutralization experiments using antibodies raised against the *Locusta* diuresin blocked both the in vitro and in vivo actions of the peptide in *Locusta* (Patel et al., 1995). These data suggest that the diuresins function as diuretic hormones in several insect groups.

The mosquito diuresins have not been extensively studied. Factor III, one of the factors isolated from head extracts of *A. aegypti*, may function through a cAMP dependent mechanism, similar to CRF-like diuretic peptides. Treatment of tubules with factor III induced changes in tubule electrophysiology, intracellular cAMP and fluid secretion similar to the CRF-like diuretic peptides, suggesting factor III represents a CRF-like peptide (Williams and Beyenbach, 1983; Petzel et al., 1985; 1986; Wheelock et al., 1988). Recently, a putative mosquito diuretic hormone has been purified and sequenced from *C. salinarius* by Dr. F. Clottens of the U.S. Department of Agriculture (Clottens, personal communication) and synthesized by the Division of Biotechnology of the University of Arizona. The amino acid sequence of this mosquito peptide closely resembles the diuresin family of neuropeptides. It is therefore likely that the *C. salinarius* diuresin would produce physiological responses similar to those found in other insects. The *C. salinarius* diuresin would likely produce a biological response because cross reactivity for other insect diuresins has previously been demonstrated (Audsley et al., 1995).

The mosquito diuresin and mosquito leucokinins exhibit strong amino acid sequence homology to the two major neuropeptide families implicated in regulating fluid secretion at the Malpighian tubules, the CRF-like diuretic peptides and myotropic leucokinins. These families probably mediate their actions at the Malpighian tubules through different intracellular signal transduction pathways. The diuresins function through a receptor mediate activation of adenylate cyclase resulting in an increase in cellular

cAMP (Kay et al., 1992; Audsley et a., 1993). The leucokinins function independently of cAMP, but require cellular Ca^{2+} (Davies et al., 1995; O'Donnell et al., 1996) suggesting that the leucokinins function through a second messenger pathway which is distinct from the diuresins. It is possible that the mosquito diuresin will increase intracellular cAMP in isolated Malpighian tubules and the mosquito leucokinins increases tubule IP_3.

The specific aim of this work was to evaluate the physiological significance of these putative mosquito diuretic peptides at different levels of biological activity. Herein we report the effect of the mosquito leucokinins, *A. aegypti* leucokinin peptides, and culekinin depolarizing peptides, and the *C. salinarius* CRF-like diuretic peptide, on in vivo urine production in the adult female yellow fever mosquito, *A. aegypti*. The inhibition of peptide stimulated urine production by the treatment of mosquitoes with antibodies raised against several of these neuropeptides is presented. At the cellular level, the effects of the mosquito leucokinins and mosquito diuresin on changes in the tubule cellular second messengers, cAMP and IP_3 are also presented.

REFERENCES

Alberts B., Brey D., Lewis J., Raff M., Roberts K. and Watson J. D. (1989) The plasma membrane. In: *Molecular Biology of the Cell.* pp. 276-337. Garland Publishing, New York.

Audsley N., Coast G. M. and Schooley D. A. (1993) The effects of *Manduca sexta* diuretic hormone on fluid transport by the Malpighian tubules and cryptonephric complex of *Manduca sexta*. *J. Exp. Biol.* **178**, 231-243.

Audsley N., Kay I., Hayes T. K. and Coast G. M. (1995) Cross reactivity studies of CRF-related peptides on insect Malpighian tubules. *Comp. Biochem. Physiol.* **110A**, 87-93.

Baines R. A. and Downer R. G. H. (1991) Pharmacological characterization of a 5-hydroxytryptamine-sensitive receptor/adenylate cyclase complex in the mandibular closer muscles of the cricket, *Gryllus domestica*. *Arch. Insect Biochem. Physiol.* **16**, 153-163.

Banner S. E., Osborne R. H. and Cattel K. J. (1987) The pharmacology of the isolated foregut of the locust *Schistocerca gregaria*. II. Characterization of a 5-HT2-like receptor. *Comp. Biochem. Physiol.* **88C**, 139-144.

Barrett M. and Orchard I. (1990) Serotonin-induced elevation of cAMP levels in the epidermis of the blood-sucking bug, *Rhodnius prolixus*. *J. Insect Physiol.* **36**, 625-633.

Berridge M. J. (1981) Electrophysiologicial evidence for the existence of separate receptor mechanisms mediating the action of 5-hydroxytryptamine. *Mol. Cell. Endocrinol.* **23**, 91-104.

Bern H. A., Pearson D., Larson B. A. and Nishioka R. S. (1985) Neurohormones from fish tailes: the caudal neurosecretory system. I. "Urophysiology" and the caudal neurosecretory systme of fishes. *Rec. Prog. Horm. Res.* **41**, 533-552.

Berridge M. J. (1966) The physiology of excretion in the cotton stainer, *Dysdercus fasciatus*, Signoret. IV. Hormonal control of excretion. *J. Exp. Biol.* **44**, 553-566.

Berridge M. J. (1983) Rapid accumulation of inositol trisphosphate reveals that agonists hydrolyse polyphosphoinositides instead of phosphatidylinositol. *Biochem. J.* **212**, 849-858.

Berridge M. J. and Heslop J. P. (1981) Separate 5-hydroxytryptamine receptors on the salivary gland of the blowfly are linked to the generation of either cyclic adenosine 3',5'-monophosphate or calcium signals. *Br. J. Pharmac.* **73**, 729-738.

Bertram G. (1989) Fluid secretion of Malpighian tubules of *Drosophila hydei* affected by amiloride--is there a K^+/H^+-antiporter? *Verh. dt. Zool. Ges.* **82**, 203-204.

Bertram G., Schleithoff L., Zimmermann P. and Wessing A. (1991) Bafilomycin-A1 is a potent inhibitor of urine formation by Malpighian tubules of *Drosophila hydei*: is a vacuolar-type ATPase involved in ion and fluid secretion? *J. Insect Physiol.* **37**, 201-209.

Blackburn M. (1991) Isolation and identification of a new diuretic peptide from the tobacco hornworm, *Manduca sexta*. *Biochem. Biophys. Res. Commun.* **181**, 927-932.

Blackburn M. B. and Ma M. C. (1994) Diuretic activity of Mas-DP II, an identified neuropeptide from *Manduca sexta*: an in vivo and in vitro examination in the adult moth. *Arch. Insect Biochem. Physiol.* **27**, 3-10.

Blackburn M. B., Wagner R. M., Shabanowitz J., Kochansky J. P., Hunt D. F. and Raina A. K. (1995) The isolation and identification of three diuretic kinins from the abdominal ventral nerve cord of adult Helicoverpa zea. *J. Insect Physiol.* **41**, 723-730.

Bonvalet J. P., Pradelles P. and Farman N. (1987) Segmental synthesis and actions of prostaglandins along the nephron. *Am. J. Physiol.* **253**, F377-F387.

Bradley T. J. (1987a) The excretory system: structure and function. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology* (Eds. Kerkut G. A. and Gilbert L. I.), pp 421-445. Pergamon Press, Oxford.

Bradley T. J. (1987b) Physiology of osmoregulation in mosquitoes. *Ann Rev Entomol.* **32**, 439-462.

Bradley T. J. and Snyder C. (1989) Fluid secretion and microvillar ultrastructure in mosquito Malpighian tubules. *Am. J. Physiol.* **257**, 1096-1102.

Bradley T. J., Nayar J. K. and Knight J. W. (1990) Selection of a strain of *Aedes aegypti* susceptible to Dinoflaria immitis and lacking intracellular concretions in the Malpighian tubules. *J. Insect Physiol.* **36**, 709-717.

Bradley T. J., Stuart A. M. and Satir P. (1982) The ultrastructure of the larval Malpighian tubules of a saline-water mosquito. *Tissue Cell* **14**, 759-773.

Cantera R., Hansson B. S., Hallberg E. and Nassel D. R. (1992) Postembryonic development of leucokinin I-immunoreactive neurons innervating a neurohemal organ in the turnip moth *Agrotis segetum*. *Cell Tissue Res.* **269**, 65-77.

Cantera R. and Nassel D. R. (1992) Segmental peptidergic innervation of abdominal targets in larval and adult dipteran insects revealed with an antiserum against leucokinin I. *Cell Tissue Res.* **269**, 459-471.

Chapman R. F. (1982) Excretion and salt and water regulation, In: *The Insects structure and function*, pp. 574-603. Harvard University Press, Cambridge, MA.

Chen Y., Veenstra J. A., Davis N. T. and Hagedorn H. H. (1994a) A comparative study of leucokinin-immunoreactive neurons in insects. *Cell Tissue Res.* **276**, 69-83.

Chen Y., Veenstra J. A., Hagedorn H. and Davis N. T. (1994b) Leucokinin and diuretic hormone immunoreactivity of neurons in the tobacco hornworm, Manduca sexta, and co-localization of this immunoreactivity in lateral neurosecretory cells of abdominal ganglia. *Cell Tissue Res.* **278**, 493-507.

Clark T. M. (1994) Ph.D. Dissertation, University of California, Irvine. Hormonal control of Malpighian tubules in larval *Aedes aegypti*.

Clark T. M. and Bradley T. J. (1996) Stimulation of Malpighian tubules from larval Aedes aegypti by secretagogues. *J. Insect Physiol.* **42**, 593-602.

Clark T. M. and Bradley T. J. (1997) Malpighian tubules of larval *Aedes aegypti* are hormonally stimulated by 5-HT in response to increased salinity. *Arch. Insect Biochem. and Physiol.* **34**, 123-141.

Clements A. N. (1992) Adult diuresis, excretion and defaecation, In: The Biology of Mosquitoes, pp 304-326. Chapman and Hall, New York.

Clottens F. L., Meola S . M. Coast G. M. Hayes T. K. Wright M. S. Nachman R. J. and Holman G. M. (1993) Characterization of an antiserum against an acetakinin I-analog and its use for the localization of culekinin depolarizing peptide II in the mosquito, *Culex salinarius*. *Regul Pept.* **49**, 145-157.

Clottens F. L., Holman G. M., Coast G. M., Totty N. F., Hayes T. K., Kay I., Mallet A. I., Wright M. S., Chung J. S., Truong O. and Bull D. L. (1994) Isolation and characterization of a diuretic peptide common to the house fly and stable fly. *Peptides* **15**, 971-979.

Coast G. M. (1988) Fluid secretion by single isolated Malpighian tubules of the house cricket, *Acheta domesticus*, and their response to diuretic hormone. *Physiol. Ent.* **13**, 381-391.

Coast G. M., Holman G. M. and Nachman R. J. (1990) The diuretic activity of a series of cephalomyotropic neuropeptides, the achetakinins, on isolated Malpighian tubules of the house cricket, *Acheta domesticus*. *J. Insect Physiol.* **36**, 481-488.

Coast G. M. and Kay I. (1994) The effects of *Acheta* diuretic peptide on isolated Malpighian tubules from the house cricket *Acheta domesticus*. *J. Exp. Biol.* **187**, 225-243.

Davies S. A., Huesmann G. R., Maddrell S. H. P., O'Donnell M. J., Skaer N. J. V., Dow J. A. T. and Tublitz N. J. (1995) CAP2b, a cardioacceleratory peptide, is present in Drosophila and stimulates tubule fluid secretion via cGMP. *Am. J. Physiol.* **269**, R1321-R1326.

Davis N. T. (1987) Neurosecretory neurons and their projections to the serotonin neurohemal system of the cockroach *Periplaneta americana* (L.) and identification of mandibular and maxillary motor neurons associated with this system. J. comp. Neurol. **259**, 604-621.

Davis N.T. and Hildebrand J.G. (1992) Vasopressin-immunoreactive neurons and neurohemal systems in cockroaches and Mantids. *J. comp. Neurol.* **320**, 381-393.

DeGuire D. M. and Fraenkel G. (1973) The meconium of Aedes aegypti (Diptera: Culicidae). *Ann. Entomol. soc. Am.* **66**, 475-476.

Diamond J. M. and Bossert W. H. (1968) Standing gradient osmotic flow. A mechanism for coupling of water and solute transport in epithelia. *J.Gen Physiol.* **50**, 2061-2083.

Digan M. E., Roberts D. N., Enderlin F. E., Woodworth A. R. and Kramer S. J. (1992) Characterization of the precursor for *Manduca sexta* diuretic hormone Mas-DH. *Proc. Natl. Acad. Sci.* **89**, 11074-11078.

DiGiovanni S. R., Nielsen S., Christensen E. I. and Knepper M. A. (1994) Regulation of collecting duct water channel expression by vasopressin in Brattleboro rat. *Proc. Natl. Acad. Sci. USA* **91**, 8984-8988.

Dijkstra S., Lohrmann E., Van Kerkhove E. and Greger R. (1994) Characteristics of the luminal proton pump in Malpighian tubules of the ant. *Renal Physiol. Biochem.* **17**, 27-39.

Dow J. (1981) Countercurrent flows, water movements and nutrient absorption in the locust midgut. *J. Insect Physiol.* **27**, 579-585.

Dow J. A. T., Kelly D. C., Davies S. A., Maddrell S. H. P. and Brown D. (1995) A novel member of the major intrinsic protein family in *Drosophila*: are aquaporins involved in insect Malpighian (renal) tubule fluid secretion? *J. Physiol.* **489**, 110P-111P.

Evans P. D. and Cournil I. (1990) Co-localization of FLRF-and vasopressin-like immunoreactivity in a single pair of sexually dimorphic neurones in the nervous system of the locust. *J. Comp. Neurology* **292**, 331-348.

Fogg K., Anstee J. and Hyde D. (1990) Effects of corpora cardiaca extract on intracellular second messenger levels in Malpighian tubules of *Locusta migratoria* L. *J. Insect Physiol.* **36**, 383-389.

Furuya K., Schegg K. M., Wang H., King D. S. and Schooley D. A. (1995) Isolation and identification of a diuretic hormone from the mealworm *Tenebrio molitor*. *Proc. Natl. Acad. Sci.* **92**, 12323-12327.

Gee J. D. (1975) The control of diuresis in the Tsetse fly *Glossina austeni*. A preliminary investigation of the diuretic hormone. *J. exp. Biol.* **63**, 391-401.

Gee J. D. (1976) Active transport of sodium by the Malpighian tubules of the Tsetse fly *Glossina morsitans*. *J. exp. Biol.* **64**, 357-368.

Gillett J. D. (1982) Diuresis in newly emerged, unfed mosquitoes. I. Fluid loss in normal females and males during the first 20 hours of adult life. Proc. R. Soc. Lond **B 216**, 201-207.

Gillett J. D. (1983) Diuresis in newly emerged, unfed mosquitoes. II. The basic pattern in relation to escape from the water, preparation for mature flight, mating and the first blood meal. *Proc. R. Soc. Lond* **B 217**, 237-242.

Gole J. W. D., Orr G. L. and Downer R. G. H. (1987) Pharmacology of octopamine, dopamine and 5-hydroxytryptamine-stimulated cyclic AMP accumulation in the corpus cardiacum of the American cockroach, *Periplaneta americana*. L. *Arch. Insect Biochem. Physiol.* **5**, 119-128.

Gupta B. L., Hall T. A., Maddrell S. H. P. and Moreton R. B. (1976) Distribution of ions in a fluid-transporting epithelium determined by electron-probe X-ray microanalysis. *Nature* **264**, 284-287.

Hadley M. E. (1988) Neurohormones, In: Endocrinology. pp. 496-527. Prentice-Hall, New Jersey.

Harvey W. R. (1992) Physiology of V-ATPase. *J. Exp. Biol.* **172**, 1-17.

Harvey W. R., Cioffi M., Dow J.A.T. and Wolfersberger M. G. (1983) Potassium ion transport ATPase in insect epithelia. *J. Exp. Biol.* **106**, 91-117.

Hayes T. K., Holman M. G., Pannabecker T. L., Wright M. S., Strey A. A., Nachman R. J., Hoel D. F., Olson J. K. and Beyenbach K. W. (1994) Culekinin depolarizing peptide: a mosquito leucokinin-like peptide that influences insect Malpighian tubule ion transport. *Regul. Pept.* **52**, 235-248.

Hayes T. K., Pannabecker T. L., Hinckley D. J., Holman G. M., Nachman R. J., Petzel D. H. and Beyenbach K. W. (1989) Leucokinins, a new family of ion transport stimulators and inhibitors in insect Malpighian tubules. *Life Sci.* **44**, 1259-1266.

Hegarty J., Zhang B., Pannabecker T., Petzel D., Baustian M. and Beyenbach K. W. (1991) Dibutyryl cAMP activates bumetanide-sensitive electrolyte transport in Malpighian tubules. *Am. J. Physiol.* **261**, C521-C529.

Hill A. (1980) Salt-water coupling in leaky epithelia. *J. Membr. Biol.* **56**, 177-182.

Hiripi L. and Downer R. G. H. (1993) Characterization of serotonin binding sites in insect (*Locusta migratoria*) brain. *Insect Biochem. Molec. Biol.* **23**, 303-307.

Holman G.M., Cook B.J. and Nachman R.J. (1986a) Isolation, primary structure and synthesis of two neuropeptides from *Leucophaea maderae:* members of a new family of cephalomyotropins. *Comp. Biochem. Physiol.* **84C**, 205-211.

Holman G. M., Cook B. J. and Nachman R. J. (1986) Isolation, primary structure and synthesis of two neuropeptides from *Leucophaea maderae*: members of a new family of cephalomyotropins. *Comp. Biochem. Physiol.* **84C**, 271-276.

Holman G. M., Cook B. J. and Nachman R. J. (1987a) Isolation, primary structure and synthesis of leucokinins V and VI. *Comp. Biochem. Physiol.* **88C**, 27-30.

Holman G. M., Cook B. J. and Nachman R. J. (1987b) Isolation, primary structure and synthesis of leucokinins VII and VIII: the final members of this new family of cephalomyotropins from head extracts of *Leucophaea maderae*. *Comp. Biochem. Physiol.* **88C**, 31-34.

Holman G. M., Nachman R. J. and Wright M. S. (1990) A strategy for the isolation of structural characterization of certain insect myotropic peptides that modify spontaneous contractions of the isolated cockroach hindgut. In: *Chromatography and Isolation of Insect Hormones and Pheromones*, (Ed. McCaffery A. R. and Wilson I. D.) pp195-204. Plenum Press, New York.

House C. R. and Ginsborg B. L. (1985) Salivary gland. In: *Comprehensive Insect Biochemistry, Physiology and Pharmacology*, (Ed. Kerdut G. A. and Gilbert L.)195-224. Pergammon Press, Oxford.

Hughes L. (1979) Further investigation of the isolation of diuretic hormone from *Rhodnius prolixus*. *Insect Biochem.* **9**, 247-255.

Ichikawa T., McMaster D., Lederis K. and Kobayashi H. (1982) Isolation and amino acid sequence of urotensin I, a vasoactive and ACTH-releasing neuropeptide, from the carp (Cyprinus carpio) urophysis. *Peptides* **3**, 859-867

Isaacson L., Nicolson S. and Fisher D. (1989) Electrophysiological and cable parameters of perfused beetle Malpighian tubules. *Am. J. Physiol.* **257**, R1190-R1198.

Kataoka H., Troetschler R., Li J., Kramer S., Carney R. and Schooley D. (1989) Isolation and identification of a diuretic hormone from the tobacco hornworm, *Manduca sexta*. *Proc. Natl. Acad. Sci.* **86**, 2976-2980.

Kay I., Coast G. M., Cusinato O., Wheeler C. H., Totty N. F. and Goldsworthy G. J. (1991a) Isolation and characterization of a diuretic peptide from *Acheta domesticus*. *Biol. Chem. Hoppe-Seyler* **372**, 505-512.

Kay I., Patel M., Coast G. M., Totty N. F., Mallet A. I. and Goldsworthy G. J. (1992) Isolation, characterization and biological activity of a CRF-related diuretic peptide from *Periplaneta americana* L. *Regul. Pept.* **42**, 111-122.

Kay I., Wheeler C. H., Coast G. M., Totty N. F., Cusinato O., Patel M. and Goldsworthy G. J. (1991b) Characterization of a diuretic peptide from *Locusta migratoria*. *Biol. Chem. Hoppe-Seyler* **372**, 929-934.

Keynes R. D. (1969) From frog skin to sheep rumen: A survey of transport of salts and water across multicelluular strctures. *Q. Rev. Biophys.* **2**, 177-281.

Klein U. (1992) The insect V-ATPase, a plasma membrane portons pump energizing secondary active transport: immunological evidence for the occurrence of a V-ATPase in insect ion transproting epithelia. *J. Exp. Biol.* **172**, 345-354.

Lange A. B. (1988) Inositol phospholipid hydrolysis may mediate the action of proctolin on insect visceral muscle. *Arch. Insect Biochem. Physiol.* **9**, 201-209.

Lange A. B., Orchard I. and Barrett F. M. (1989) Changes in haemolymph serotonin levels associated with feeding in the blood-sucking bug, *Rhodnius prolixus*. *J. Insect Physiol.* **35**, 393-399.

Lange A. B., Orchard I. and Lloyd R. J. (1988) Immunohistochemical and electrochemical detection of serotonin in the nervous system of the blood-feeding bug, *Rhodnius prolixus*. *Archs. Insect Biochem. Physiol.* **8**, 188-201.

Lehmberg E., Ota R., Furuya K., King D., Applebaum S., Ferenz H. and Schooley D. (1991) Indentification of a diuretic hormone of *Locusta migratoria*. *Biochem. Biophys. Res. Commun.* **179**, 1036-1041.

Lehmberg E., Schooley D. A., Ferenz H. J. and Applebaum S. W. (1993) Characteristics of *Locusta migratoria* diuretic hormone. *Arch. Insect Biochem. Physiol.* **22**, 133-140.

Leyssens A., Kerkhove E. V. and Steels P. (1991) Basolateral electrochemical K+ gradients in Malpighian tubules of Formica in different transport rate conditions. *Pflugers Arch* **418**, R72.

Leyssens A., Steels P., Lohrmann E., Weltens R. and Van Kerkhove E. (1992) Intrinsic regulation of potassium transport in Malpighian tubules of Formica. Electrophysiological evidence. *J. Insect Physiol.* **38**, 431-446.

Leyssens A., Zhang S. L., Weltens R., Van Kerdhove E. and Steels P. (1991) Evidence for the presence of an electrogenic cation pump in the isolated Malpighian tubules of *Formica*. *Arch. Int. Physiol.* Biochem. **99**, P10.

Loeb M. J. (1993) Hormonal control of growth and reproduction in the arthropods: introduction to the symposium. *Am. Zool.* **33**, 303-307.

Maddrell S. H. P. (1963) Excretion in the blood-sucking bug *Rhodnius prolixus* Stal. I. The control fo diuresis. *J. exp. Biol.* **40**, 247-256.

Maddrell S. (1980) Characteristics of Epithelial transport in insect Malpighian tubules. *Currrent Topics in Membranes and Transport* **14**, 427-463.

Maddrell S. H. P. and O'Donnel M. J. (1992) Insect Malpighian tubules: V-ATPase action in ion and fluid transport. *J. Exp. Biol.* **172**, 417-429.

Maddrell S. H. P. and Overton J. A. (1988) Stimulation of sodium transport and fluid secretion in an insect Malpighian tubule. *J. Exp. Biol.* **137**, 265-276.

Maddrell S. H. P. and Phillips J. E. (1975) Regulation of absorption in insect excretory systems. In: *Perspectives in Experimental Biology, vol I, Zoology* (Ed. Davies P. S) pp 179-185. Pergamon Press, Oxford.

Maddrell S. H. P., Pilcher D. E. M. and Gardiner B. O. D. (1969) Stimulatory effect of 5-hydroxytryptamine (serotonin) on secretion by Malpighian tubules of insects. *Nature* **222**, 784-785.

Maddrell S. H. P., Pilcher D. E. M. and Gardiner B. O. D. (1971) Pharmacology of the Malpighian tubules of *Rhodnius* and *Carausius*: the structure-activity relationship of tryptamine analogues and the role of cyclic AMP. J. exp. Biol. **54**, 779-804.

Maddrell S. H. P., Herman W. S., Mooney R. L. and Overton J. A. (1991) 5-Hydroxytryptamine: a second diuretic hormone in *Rhodnius prolixus*. *J. Exp. Biol.* **156**, 557-566.

Mathew G. and Rai K. S. (1976) Fine structure of the Malpighian tubule of *Aedes aegytpi*. *Ann. Entomol. Soc. Am.* **69**, 659-661.

Mills R. R. (1967) Hormonal control of excretion in the American cockroach-I. Release of a diuretic hormone from the terminal abdominal ganglion. *J. Exp. Biol.* **46**, 35-41.

Montecucchi P. C. and Henschen A. (1981) Amino acid composition and sequence analysis of sauvagin, a new active peptide from the skin of *Phyllomedusa sauvagei*. Int. *J. Peptide Res.* **18**, 113-120.

Morgan P. J. and Mordue W. (1984) 5-Hydroxytryptamine stimulates fluid secretion in locust Malpighian tubules independently of cAMP. *Comp. Biochem. Physiol.* **79C**, 305-310.

Morgan P. J. and Mordue W. (1985a) Cyclic AMP and locust diuretic hormone action. *Insect Biochem.* **15**, 247-257.

Morgan P. J and Mordue W. (1985b) The role of calcium in diuretic hormone action on locust Malpighian tubules. *Mol. Cell. Endocrinol.* **40**, 221-231.

Nachman R. J., Coast G. M., Holman G. M. and Beier R. C. (1995) Diruetic activity of C-terminal group analoques of the insect kinins in *Acheta domesticus*. *Peptides* **16**, 809-813.

Nachman R. J., Coast G. M., Holman M. G. and Haddon W. F. (1992) A bifunctional heterodimeric insect neuropeptide analog. *Int. J. Peptide Protein Res.* **40**, 423-428.

Nassel D. R. and Elkes K. (1985) Serotonergic terminals in the neural sheath of the blowfly nervous system: electron microscopical immunocytochemistry and 5,7-dihydroxytryptamine labelling. *Neuroscience.* **1**, 292-307.

Nassel D. R., Cantera R. and Karlsson A. (1992) Neurons in the cockroach nervous system reacting with antisera to the neuropeptide leucokinin I. *J Comp Neurol* **322**, 45-67.

Nicolson S. W. (1976) The hormonal control of diuresis in the cabbage white butterfly, *Pieris brassicae. J. Exp. Biol.* **65**, 669-683.

Nicolson S. W. (1991) Diuresis or clearance: is there a physiological role for the "diuretic hormone" of the desert beetle *Onymacris*? *J. Insect Physiol.* **37**, 447-452.

Nicolson S. W. (1993) The ionic basis of fluid secretion in insect Malpighian tubules: advances in the last ten years. *J. Insect Physiol.* **39**, 451-458.

Nicolson S. W. and Hanrahan S. A. (1986) Diuresis in a desert beetle? Hormonal control of the Malpighian tubules of *Onymacris plana* (Coleoptera: Tenebrionidae). *J. comp. Physiol. B* **156**, 497-413.

Nicolson S. and Isaacson L. (1987) Transepithelial and intracellular potentials in isolated Malpighian tubules of tenebrionid beetle. *Am. J. Physiol.* **252**, F645-F653.

Nicolson S. and Isaacson L. (1990) Patch clamp of the basal membrane of beetle Malpighian tubules: direct demonstration of potassium channels. *J. Insect Physiol.* **36**, 877-884.

Nicolson S. W. and Millar R. P. (1983) Effects of biogenic amines and hormones on butterfly Malpighian tubules: dopamine stimulates fluid secretion. *J. Insect Physiol.* **29**, 611-615.

Nijhout H. F. and Carrow G. M. (1978) Diuresis after a bloodmeal in female *Anopheles freeborni. J. Insect Physiol.* **24**, 293-298.

Novak M. G., Ribeiro J. M. C. and Hildebrand J. G. (1995) 5-hydroxytryptamine in the salivary glands of adult female Aedes aegypti and its role in regulation of salivation. *J. Exp. Biol.* **198**, 167-174.

Ochard I., Lange A. B. and Barrett F. M. (1988) Serotonergic supply to the epidermis of *Rhodnius prolixus*: evidence for serotonin as the plasticising factor. *J. Insect Physiol.* **34**, 873-879.

O'Donnell M. J., Dow J. A. T., Huesmann G. R., Tublitz N. J. and Maddrell S. H. P. (1996) Separate control of anion and cation transport in Malpighian tubules of *Drosophila melanogaster. J. Exp. Biol.* **199**, 1163-1175.

Pannabecker T. L., Aneshansley D. J. and Beyenbach K. W. (1992) Unique electrophysiological effects of dinitrophenol in Malpighian tubules. *Am. J. Physiol.* **263**, R609-R614.

Pannabecker T. L., Hayes T. K. and Beyenbach K. W. (1993) Regulation of epithelial shunt conductance by the peptide leucokinin. *J. Membr. Biol.* **132**, 63-76.

Patel M., Hayes T. K. and Coast G. M. (1995) Evidence for the hormonal function of a CRF-related diuretic peptide (*Locusta* DP) in *Locusta migratoria*. *J. Exp. Biol.* **198**, 793-804.

Patel M., Chung J. S., Kay I., Mallet A. I., Gibbon C. R., Thompson K. S. J., Bacon J. P. and Coast G. M. (1994) Localization of Locusta-DP in locust CNS and hemolymph satifsies initial hormonal criteria. *Peptides* **15**, 591-602.

Petzel D. (1985) Preliminary isolation of mosquito natriuretic factor. *Am. J. Physiol.* **249**, R379-R386.

Petzel D. H. and Samuelson D. (1992) Inhibition of eicosaniod biosynthesis modulates basal fluid secretion in the Malpighian tubules of the yellow fever mosquito (*Aedes aegypti*). *J. Insect Physiol.* **38**, 1-8.

Petzel D. H, Hagedorn H. H. and Beyenbach K. W. (1986) Peptide nature of two mosquito natriuretic factors. *Am. J. Physiol.* **250**, R328-R332.

Petzel D. H., Berg M. and Beyenbach K. W. (1987) Hormone-controlled cAMP-mediated fluid secretion in yellow-fever mosquito. *Am. J. Physiol.* **253**, R701-R711.

Petzel D. H., Parrish A. K., Ogg C. L., Witters N. A., Howard R. W. and Stanley-Samuelson D. W. (1993) Arachidonic acid and protaglandin E2 in Malpighian tubules of female yellow fever mosquitoes. *Insect Biochem. Molec. Biol.* **23**, 431-437.

Phillips J. E. (1983) Endocrine control of salt and water balance: excretion. In: *Endocrinology of Insects*, (Ed. Downer R. G. H. and Laufer H.), pp 411-425. A. R. Liss, New York.

Phillips J. E., Hanrahan J., Chamberlin M. and Thomson B. (1986) Mechanisms and control of reabsorption in insect hindgut. *Adv. Insect Physiol.* **19**, 329-422.

Picquot M. and Proux J. (1988) Relationship between excretion of primary urine and haemolymphatic level of diuretic hormone along a cercadian cycle in the migratory locust. *Physiol. Ent.* **13**, 455-460.

Proux J. P., Delaage M. P. and Chauveau J. (1987a) Immunoaffinity purification of a diuretic hormone from the nervous ventral cord of the migratory locust. *Comp. Biochem. Physiol.* **88B**, 807-811.

Proux J. P., Rougon G. and Cupo A. (1982) Enhancement of excretion across locust Malpighian tubules by a diuretic vasopressin-like hormone. *Gen. Comp. Endocr.* **47**, 449-457.

Proux J. P., Miller C. A., Li J. P., Carney R. L., Girardie A., Delaage M. P. and Schooley D. A. (1987b) Identification of an arginine vasopressin-like diuretic hormone from *Locusta migratoria*. *Biochem. Biophys. Res Comm.* **149**, 180-186.

Ramsay J. A. (1952) The excretion of sodium and potassium by the Malpighian tubules of *Rhodnius*. *J. exp. Biol.* **29**, 110-126.

Reagan J. D. (1994) Expression cloning of an insect diuretic hormone receptor. *J. Biol. Chem.* **261**, 9-12.

Reagan J. D., Miller W. and Kramer S. (1992) Allatotropin-induced formation of inositol phosphates in the corpora allata of the moth *Manduca sexta*. *Arch. Insect Biochem. Physiol.* **20**, 145-155.

Robertson B. L., Athar S. and Shelton R. L. (1977) Osmotic control of vasopressin function. In: Disturbances In Body Fluid Osmolality. pp. 125-148. Washington D.C.: Am. Physiol. Soc.

Russell V. E. W., Klein U., Reuveni M., Spaeth D. D., Wolfersberger M. G. and Harvey W. R. (1992) Antibodies to mammalian and plant V-ATPases cross react with the V-ATPase of insect cation-transporting plasma membranes. *J. Exp. Biol.* **166**, 131-143.

Saudou F., Boschert U., Amlaiky N., Plassat J. L. and Hen R. (1992) A family of Drosophila serotonin receptors with distinct intracellular signalling properties and exptression patterns. *EMBO J.* **11**, 7-17.

Sawyer D. and Beyenbach K. W. (1985) Dibutyryl-cAMP increases basolateral sodium conductance of mosquito Malpighian tubules. *Am. J. Physiol.* **17**, R339-R345.

Schoofs L., Holman G. M., Proost P., Damme J. V., Hayes T. K. and Loof A. D. (1992) Locustakinin, a novel myotropic peptide from *Locusta migratoria*, isolation, primary structure and synthesis. *Regul. Pept.* **37**, 49-57.

Schooley D. A. (1993) Insect diuretic hormones with homology to sauvagine/CRF/urotensin I. In: Molecular Comparative Physiology. (Ed. Beyenbach K. W.), pp 22-37. Karger, Basel.

Schooley D. A., Miller C. A. and Proux J. P. (1987) Isolation of two arginine vasopressin-like factors from ganglia of *Locusta migratoria*. *Archs. Insect Biochem. Physiol.* **5**, 157-166.

Schwartz L. M. and Reynolds S. E. (1979) Fluid transport in *Calliphora* Malpighian tubules: a diuretic hormone from the thoracic ganglion and abdominal nerves. *J. Insect Physiol.* **25**, 847-854.

Schweikl H., Klein U., Schindbeck M. and Wieczorek H. (1989) A vacuolar-type ATPase, partially purified from potassium transporting plasma membranes of tobacco hornworm midgut. *J. Biol. Chem.* **264**, 11136-11142.

Shibanaka Y., Hayashi H., Takai M. and Fujita N. (1993) Eclosion hormone activates phosphatidylinositol hydrolysis in silkworm abdominal ganglia during adult metamorphosis. *Eur. J. Biochem.* **211**, 427-430.

Spring J. H. and Clark T. M. (1990) Diuretic and antidiuretic factors which act on the Malpighian tubules of the house cricket, *Acheta domesticus*. *Progress in Comparative Endocrinology* **9**, 559-564.

Stobbart R. H. and Shaw J. (1974) Salt and water balance: excretion. In: *The Physiology of Insecta*, (Ed. by Rockstein M.), pp. 362-446. Academic Press, New York.

Thompson K. S. J., Tyrer N. M., May S. T. and Bacon J. P. (1991) The vasopressin-like immunoreactive (VPLI) neurons of the locust, *Locusta migratoria*: I. Anatomy. *J. Comp. Physiol.* **168**, 605-617.

Thompson K. S. J., Rayne R. C., Gibbon C. R., May S. T., Patel M., Coast G. M. and Bacon J. P. (1995) Cellular colocalization of diuretic peptides in locusts: a potent control mechanism. *Peptides* **16**, 95-104.

Troetschler R. and Kramer S. (1992) Mode of action studies on a *Manduca sexta* diuretic hormone. *Arch. Insect Biochem. Physiol.* **20**, 35-47.

Tublitz N. J. (1988) Insect cardioactive neuropeptides: peptidergic modulation of the intrinsic ryhthm of an insect heart is mediated by inositol 1,4,5-trisphosphate. *J. Neurosci.* **8**, 4394-4399.

Tublitz N. J. and Trombley P. Q. (1987) Peptide action on insect cardiac muscle is mediated by inositol trisphosphate. *Soc. Neurosci.* Abstr **13**, 235.

Vale W., Spiess J., Rivier J. (1981) Characterization of a 41-residue bovine hypothalamic peptide that stimulates secretion of corticotropin and B-endorphin. *Science* **213**, 1394-1397.

Van Kerkhove E. (1994) Cellular mechanisms of salt secretion by the Malpighian tubules of insects. *Belg. J. Zool.* **124**, 73-90.

Veenstra J. (1988) Effects of 5-hydroxytryptiamine on the Malpighian tubules of *Aedes aegypti*. *J. Insect Physiol.* **34**, 299-304.

Veenstra J. A. (1994) Isolation and identification of three leucokinins from the mosquito *Aedes aegypti*. *Biochemical Biochemical and Biophysical Research Communications* **202**, 715-719.

Veenstra J. A. and Hagedorn H. H. (1991) Indentification of neuroendocrine cells producing a diuretic hormone in the tobacco hornworm moth, *Manduca sexta*. *Cell Tissue Res.* **266**, 359-364.

Wall B. J. and Ralph C. L. (1964) Evidence for hormonal regulation of Malpighian tubule excretion in the insect *Periplaneta americana* L. *Gen. Comp. Endocr.* **4**, 452-456.

Weltens R., Leyssens A., Zhang S. L., Lohrmann E., Steels P. and Van Kerkhove E. (1992) Unmasking of the apical electrogenic H+ pump in isolated Malpighian tubules (*Formica polyctena*) by the use of barium. *Cell Physiol. Biochem.* **2**, 101-116.

Wenning A., Greisinger U. and Proux J. P. (1991) Insect-like characteristics of the Malpighian tubules of a non-insect fluid secretion in the centipede *Lithobus forficatus* (Myrapoda: Chilopoda). *J. Exp. Biol.* **158**, 165-180.

Wessing A., Hevert F. and Ronnau K. (1986) Ion transport and intracellular activity of ions in Malphighian tubules of *Drosphila hydei*. *Zool. Beitr. N. F.* **30**, 297-314.

Wheelock G., Petzel D., Gillett J. D., Beyenbach K. W. and Hagedorn H. H. (1988) Evidence for hormonal control of diuresis after a blood meal in the mosquito *Aedes aegypti*. *Arch. Insect Biochem. Physiol.* **7**, 75-89.

Wieczorek H., Weerth S., Schindlbeck M and Klein U. (1989) A vacuolar-type proton pump in a vesicle fraction enriched with potassium transporting plasma membranes from tobacco hornworm midgut. *J. Biol. Chem.* **264**, 11143-11148.

Williams J. C. and Beyenbach K. W. (1983) Differential effects of secretagogues on Na and K secretion in the Malpighian tubules of *Aedes aegypti* (L.). *J. Comp. Physiol.* **149**, 511-517.

Williams J. C. and Beyenbach K. W. (1984) Differential effects of secretagogues on the electrophysiology of the Malpighian tubules of the yellow fever mosquito. *J. Comp. Physiol.* **154**, 301-309.

Worden M. K. and O'Shea M. (1986) Evidence for stimulation of muscle phosphatidylinositol metoabolism by an identified skeletal motoneuron. *Neurosci* Abst **12**, 948.

Wright J. M. and Beyenbach K. W. (1987) Chloride channels in apical membranes of mosquito Malpighian tubules. *Fed. Proc.* **46**, 1270.

Yamamoto K., Chadarevian A. and Pellegrini M. (1988) Juvenile hormone action mediated in male accessory glands of *Drosophila* by calcium and kinase C. *Science* **239**, 916-919.

2. THE EFFECT OF PUTATIVE DIURETIC FACTORS ON IN VIVO URINE PRODUCTION IN THE MOSQUITO, *AEDES AEGYPTI*

Craig Cady and Henry H. Hagedorn
The Center for Insect Science of the University of Arizona, Tucson, Arizona, USA
The Department of Entomology, 410 Forbes Bldg. 36, University of Arizona, Tucson, Arizona, USA

Abstract--Changes in total urine production were measured in the mosquito, *Aedes aegypti*, following injection of 5-hydroxytryptamine (5-HT) or the putative mosquito diuretic peptides, *C. salinarius* diuresin and mosquito leucokinins, the culekinin depolarizing peptides (CDP-I, II and III) and *A. aegypti* leucokinin peptides (ALP-I, II and III). The mosquito diuresin, leucokinins and 5-HT stimulated total urine production in a dose dependent manner. The ED_{50} for 5-HT in urine production experiments was nearly 100 fold higher than other diuretic agonists. Doses greater than 2×10^{-4} μmoles inhibited urine production, suggesting either the occurrence of receptor down regulation or that more than one type of 5-HT receptor may be active. The ALPs had relatively lower ED_{50} values, suggesting that the endogenous peptides may have higher receptor binding affinities, compared to the CDPs. Immunization of mosquitoes with polyclonal antisera raised against either ALP-I or *C. salinarius* diuresin resulted in significant inhibition of peptide stimulated urine production. The evidence presented above suggests that mosquito leucokinins and the *C. salinarius* diuresin may function in the neuroendocrine regulation of urine production in the mosquito.

INTRODUCTION

Haematophagous insects, such as the female adult yellow fever mosquito, *Aedes aegypti*, are able to ingest large meals of up to 10 times their body volume. Water, solute and nutrients from the blood meal are absorbed across the midgut epithelia producing profound changes in the ionic strength and composition of the hemolymph. In order to maintain the physiological composition of the hemolymph, a diuresis, or rapid, selective movement of water and solute from the hemolymph into the Malpighian tubules is required for the production of the final urine. The diuresis after the blood meal can reach a peak rate

of about 5 urine droplets/minute (50 nl/min), resulting in a loss of 40% of the ingested plasma volume within the first hour after the blood meal (Williams et al., 1983). In the mosquito, two powerful groups of epithelial cells function together to produce the final urine, the Malpighian tubules, the primary tissue which transports solute and water during diuresis, and the rectum, which modifies the urine prior to excretion by selective absorption of solute and water (Bradley, 1985).

Currently, two families of neuropeptides have been found to stimulate fluid secretion in the Malpighian tubules of insects, the corticotropin-releasing factor-like diuretic hormones (CRF-like DH) also known as the diuresins and the leucokinins, which were originally isolated from *Leucophaea maderae* (Holman et al., 1986a, b; 1987a, b). The diuresins exhibit amino acid sequence similarities to the CRF-like diuretic hormone family of peptides. The diuresins have been isolated from several insect species including two from *Manduca sexta* (Kataoka et al., 1989; Blackburn et al., 1991), *Acheta domesticus* (Kay et al., 1991a), *Locusta migratoria* (Lehmberg et al., 1991; Kay et al., 1991b), *Periplaneta americana* (Kay et al., 1992), *Musca domestica* and *Stomoxys calcitrans* (Clottens et al., 1994) and *Tenebrio molitor* (Furuya et al., 1995). *M. sexta* diuresin immunoreactivity has been identified in neurosecretory cell axon terminals in the corpora cardiaca of *M. sexta* (Veenstra and Hagedorn, 1991; Blackburn et al., 1991). In *L. migratoria*, a diuresin has been identified in the corpora cardiaca, abdominal ganglia and hemolymph (Patel et al., 1994; Thompson et al., 1995). A cDNA for the CRF-like diuretic hormones has been isolated in *M. sexta* and was found to encode for the precursor form of this peptide (Digan et al., 1992).

It has been suggested that the diuresins stimulate the movement of solute and water across the Malpighian tubules via a 3',5'-cyclic adenosine monophosphate (cAMP) dependent mechanism (Kay et al., 1991a, b; Kay et al., 1992, Lehmberg et al., 1991; Audsley et al., 1993). The diuresins have been shown to increase intracellular cAMP and fluid secretion in isolated Malpighian tubules in several insect species including *A. domesticus*, *M. sexta*, *L. migratoria* and *M. domesticus* (Kay et al., 1991a; Audsley et al., 1993; Clottens et al., 1994; Coast and Kay, 1994; Patel et al., 1994; Thompson et al., 1995). In vivo bioassays have also demonstrated biological activity of the diuresins in some of these insects (Kataoka et al., 1989; Blackburn et al., 1991; Patel et al., 1995).

The leucokinins, originally found to have a myotropic action at the hindgut have also been shown to increase fluid production in isolated Malpighian tubules (Hayes et al., 1989; Coast et al., 1990). Leucokinins have been isolated and sequenced from several

insects including locusts (Schoofs et al., 1992), crickets (Holman, 1990) and moths (Blackburn et al., 1995). Leucokinin immunoreactive neurosecretory cells have been identified from *M. sexta*, *A. domesticus*, *Schistocerca americana*, *Apis mellifera*, *Agrotis segetum*, *L. maderae* and *A. aegypti* (Chen et al., 1994a; Chen et al., 1994b; Thompson et al., 1995; Cantera et al., 1992; and Nassel et al., 1992).

In the mosquito, the post blood meal diuresis is probably regulated by neuroendocrine factors acting primarily at the Malpighian tubules (Wheelock et al 1988; Pannabecker, 1995). Three fractions, isolated from head extracts of the mosquito *A. aegypti*, influence Malpighian tubule physiology (Williams and Beyenbach, 1983).

There is little evidence of the diuresins in the mosquito. Fraction III, one of the fractions isolated from *A. aegypti* head extracts, may function in a similar manner as the diuresin peptides via a cAMP dependent mechanism. Treatment with fraction III induced changes in tubule electrophysiology, intracellular cAMP concentrations and fluid secretion similar to the diuresin peptide induced changes (Williams and Beyenbach, 1983; Petzel et al., 1985; 1986; Wheelock et al., 1988). Recently, a putative mosquito diuretic hormone has been purified and sequenced from *Culex salinarius* by Dr. F. Clottens of the U.S. Department of Agriculture (Clottens, personal communication) and synthesized by the Division of Biotechnology of the University of Arizona. The amino acid sequence of this *C. salinarius* peptide closely resembles the diuresin family of neuropeptides.

The leucokinins may influence tubule physiology during diuresis in the mosquito. In *A. aegypti*, the leucokinins stimulate fluid secretion in isolated tubules and have been shown to depolarize Malpighian tubule transepithelial voltage in a chloride-dependent manner (Hayes et al., 1989; Pannabecker et al., 1993; Hayes et al., 1994). Fraction II, from the *A. aegypti* head extracts, increased urine production when injected into mosquitoes (Wheelock et al., 1988). Fraction II also increased fluid secretion in isolated tubules in a chloride-dependent manner and depolarized tubule transepithelial voltage (Petzel et al., 1985), suggesting a leucokinin-like factor. Leucokinins have been isolated from two species of mosquitoes. Culekinin depolarizing peptides (CDPs) have been isolated from *C. salinarius* (Hayes et al., 1994) and *A. aegypti* leucokinin peptides (ALPs) have been isolated from *A. aegypti* (Veenstra, 1994). Recently, a cDNA was isolated from *A. aegypti* that was found to encode the preproleucokinin, indicating that the three ALPs are represented by a single gene (Veenstra et al., in press).

To evaluate the physiological significance of these putative mosquito diuretic peptides we report the effect of the mosquito leucokinins, ALPs and CDPs and the *C.*

salinarius diuresin peptide on in vivo urine production in the adult female yellow fever mosquito, *A. aegypti*. We also report the inhibition of peptide stimulated urine production by the treatment of mosquitoes with antibodies raised against two of these neuropeptides.

MATERIAL AND METHODS

Animals

Mosquitoes, *A. aegypti* (Rock strain) were raised either in small numbers as needed for individual experiments as described (Shapiro and Hagedorn, 1982), or in large numbers in shallow pans containing 3,000 larvae (Wheelock et al., 1991). Mosquitoes were maintained at 27° C on 3% sucrose solution in 16L:8D photoperiod. Animals used in experiments were non-blood fed, adult, female mosquitoes, collected 3-7 days after ecolsion and dehydrated for 12 hours prior to experiments.

In vivo injection and urine production bioassay

An in vivo urine production bioassay based on the method of Wheelock et al., (1988) was modified to determine the effects of putative diuretic factors on mosquito urine production.

Dilution of radiolabeled water and preparation of the injection syringe

Tritiated water (Amersham Corporation, Arlington Heights, IL) with a specific activity of 5 mCi/ml was diluted with *Aedes* saline to 1 X 10^8 disintegration's per minute (dpm)/ml (5 X 10^4 dpm/injection). Injection needles were made from capillary glass tubing using a Micro-pipette puller MI (Industrial Science Associates, Ridgewood, NY). Needles were attached with wax to a gas tight, microliter syringe (Hamilton, Reno, NV) and filled with light mineral oil. Diluted tritiated water in *Aedes* saline, with or without agonist was drawn into the needle. The injection solution was sealed between layers of mineral oil to prevent evaporation and release of volatile radioactive water before and between injections. Experiments using radiolabeled water were performed in a fume hood.

Blood feeding and mosquito injection

Mosquitoes were blood fed on whole bovine blood supplemented with 2 mg/ml L-isoleucine and 1.3 mg/ml adenosine triphosphate (ATP) to enhance feeding. Mosquitoes were fed blood through a thin layer of stretched parafilm over a water jacketed glass vessel warmed to 37° C. Approximately one minute after engorging, the mosquitoes were cold anesthetized on ice and decapitated using a fine forceps. Neck wounds on the thorax of decapitated mosquitoes were sealed with melted paraffin to prevent leaking. Mosquitoes were then cold anesthetized and restrained lateral side down, under fine mesh nylon net on a chilled glass microscope slide. Mosquitoes were injected intrathoracically with 0.5 μl of 5.0 X 10^4 dpm of tritiated water in saline with or without test peptide, using a micro manipulator (Brinkman Instrument Co., Germany).

Injected mosquitoes were maintained in sealed glass scintillation vials at 28° C for 15 minutes in a humidity chamber. Following incubation, vials were frozen rapidly in liquid nitrogen, the mosquito removed and placed into a new vial. The frozen urine remained in the original vial. A volume of 4.0 ml of scintillation fluid (Scintiverse Bio HP, Fisher Scientific, Fair Lawn, NJ) was dispensed into each vial and immediately mixed by rapid vortex. To determine dpm, vials were counted against a prepared quench curve in a liquid scintillation counter (Beckman Instruments Inc., Fullerton, CA) equipped with an external standard. Urine production was calculated by dividing the dpms in the collected urine by the total amount of radioactive water injected and expressed as a percent of urine collected. Urine production data from peptide-treated mosquitoes were compared with control animals treated only with saline-diluted radiolabeled water.

Recovery of radiolabeled water

To estimate the recovery of radiolabeled water from injected mosquitoes, intact mosquitoes were blood fed, decapitated, wax sealed and injected as described above. Following injection, mosquitoes were held in closed scintillation vials for 15 minutes to collect urine and subsequently rapidly frozen and transferred into new vials as above. Radioactivity was measured, as above, in vials containing collected urine or mosquitoes. The dpms from the vials containing urine were added to the dpms from vials containing mosquitoes and this sum was defined as the injected dpms (Wheelock et al., 1988).

To determine percent recovery, the final injected dpms were compared to the total count dpms. The total count dpms were determined by dispensing saline-diluted radiolabeled water below oil, using the mosquito injection apparatus. This mixture was

transferred to a scintillation vial and counted. Comparing the final injected dpms to the total count dpms gave an estimate of the percent recovery of the radiolabeled water which was injected into each mosquito. The average percent recovery was 97.1 ± 2.1 % (n = 10 mosquitoes) indicating nearly all the injected radiolabeled water was recovered during incubation and transfer of mosquitoes.

Optimal incubation time following injection

Preliminary experiments using *A. aegypti* leucokinin I (ALP-I) (see Table 2.1) were done to determine the appropriate incubation time needed to resolve differences in urine production between peptide treated mosquitoes versus control, saline treated mosquitoes. The urine production ratio was determined from the percent urine collected for peptide treated mosquitoes divided by percent urine collected for saline treated mosquitoes. The largest urine production ratio occurred following a 5 minute incubation period (Table 2.1). A 15 minute incubation was chosen because this interval enabled a more efficient handling of multiple mosquitoes during the bioassay.

These data enabled a determination of the length of time during which the injected peptide influenced urine production. The urine production ratio was substantially reduced

Table 2.1. A comparison with time of % urine collected in peptide treated mosquitoes versus control, saline treated mosquitoes

Incubation time (minutes)	Saline treated urine collected (%) ± SEM	ALP-I treated urine collected (%) ± SEM	n	Ratio % urine collected (peptide/saline)
1	0.4 ± 0.1	0.5 ± 0.12	6	1.3
5	0.4± 0.1	3.0 ± 0.3	6	7.5
15	3.2± 0.7	10.1± 2.0	6	3.2
30	5.0 ± 1.3	10.1 ± 1.7	6	2.2
60	6.2 ± 1.0	11.6 ± 1.6	6	1.9

Peptide injected ALP-I (1.6×10^{-4} M/L, 0.16 mg/ml)
Ratio % urine collected equals % urine collected from peptide treated mosquitoes/% urine collected from saline treated mosquitoes
Means are ± SEM
n equals the number of mosquitoes injected

60 minutes following injection, equivalent to that following a one minute incubation (Table 2.1). The 15 minute incubation time is within the temporal range in which an agonist could influence urine production and was therefore considered appropriate for use in this bioassay.

Immunoneutralization experiments

Urine production experiments were modified to determine if antibodies raised against putative diuretic peptides could be used in vivo to inhibit peptide stimulated increases in urine production.

Injection method. Intact mosquitoes were injected on the lateral thorax as described above with 0.2 μl of either anti *C. salinarius* diuresin at a 1:5 dilution or anti *A. aegypti* leucokinin I at a 1:10 dilution. Control mosquitoes were treated with 0.2 μl of appropriately diluted normal rabbit serum (NRS). Antibody and NRS solutions were diluted using *Aedes* saline.

After 16-18 hours to allow the initial injection site to heal, antibody or NRS treated mosquitoes were blood fed, decapitated and their neck wounds wax sealed. Radiolabeled water in saline containing either ALP-I (2.0×10^{-7} M/L, 0.4 μg/ml) or diuresin (2×10^{-6} M/L, 10.5 μg/ml) was injected into the contralateral thorax. Control mosquitoes were injected with 0.5 μl radiolabeled water diluted in saline without peptide and represented baseline controls. Following the second injection mosquitoes were maintained in scintillation vials for 15 minutes and processed as described in the in vivo injection and urine production bioassay. Urine production data from NRS/peptide treated and antibody/peptide treated were statistically compared.

It was necessary to determine if treating mosquitoes with diluted NRS for the first injection would influence urine production compared to saline treated mosquitoes. Mosquitoes were prepared and injected in a similar manner as described above. Following urine collection and counting, the NRS treated mosquitoes had urine volumes comparable to that of the saline treated mosquitoes (1.6 ± 0.2 % versus 2.0 ± 0.6 %, n = 8 mosquitoes/group). Injection of NRS does not significantly alter urine production in our bioassay. Experiments were completed in which urine production was compared for antibody treated versus saline treated mosquitoes and results were similar to the data described above.

Aedes saline

Aedes saline was prepared according to Hagedorn et al., (1977) and consisted of 150 mM/L NaCl, 3.4 mM/L KCl, 1.8 mM/L $NaHCO_3$, 0.6 mM/L $MgCl_2$, 25 mM/L Hepes and adjusted to pH 6.8 - 7.0 with 0.5N NaOH.

Antibodies

Antibodies raised in rabbits against the C-terminal portion of the *C. salinarius* diuresin peptide and ALP-I were provide by Dr. Jan Veenstra. Both antibodies were used in separate ELISAs to generate a standard curve using synthetic peptide (personal communication Dr. Jan Veenstra).

Chemicals

Culekinin depolarizing peptides were a gift from Dr. Timothy Hayes, *A. aegypti* leucokinin peptides were provided by Dr. Jan Veenstra (Table 2.2) and C. salinarius diuresin was synthesized by Dr. Ron Jasensky (Division of Biotechnology of the Arizona Research Laboratories, University of Arizona, Tucson, AZ) based on the amino acid sequence for the *C. salinarius* peptide (personal communication Dr. Frank Clottens, Food Animal Protection Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Route 5, Box 810, college Station, TX 77845), Table 2.3. All other chemicals were purchased from Sigma Chemical (St. Louis, MO).

Statistical Analysis

Statistical significance was determined with the Student's t-test using either Excel (Microsoft Corporation, Redmond, WA) or StatView (BrainPower Inc., Calabasas, CA) software. ED_{50} values for dose response curves were determined using a linear curve fit analysis.

Table 2.2. The amino acid sequences of the leucokinin related family of peptides

Isolated from, *Leucophaea maderae*	
leucokinin I	D P A **F** N **S** **W** **G-amide**
leucokinin II	D P G **F** S **S** **W** **G-amide**
leucokinin III	D Q G **F** N **S** **W** **G-amide**
leucokinin IV	D A S **F** H **S** **W** **G-amide**
leucokinin V	G S G **F** S **S** **W** **G-amide**
leucokinin VI	E S S **F** H **S** **W** **G-amide**
leucokinin VII	D P A **F** S **S** **W** **G-amide**
leucokinin VIII	G A S **F** Y **S** **W** **G-amide**
Leucokinin-related peptides isolated from, *Culex salinarius*	
Culex leucokinin I	N P **F** H **S** **W** **G-amide**
Culex leucokinin II	N N A N V **F** Y **P** **W** **G-amide**
Culex lecuokinin III	W K Y V S K Q K **F** F **S** **W** **G-amide**
Leucokinin-related peptides isolated from, *Aedes aegypti*	
Aedes leucokinin I	N S K Y V S K Q K **F** Y **S** **W** **G-amide**
Aedes leucokinin II	N P **F** H A **W** **G-amide**
Aedes leucokinin III	N N P N V **F** Y **P** **W** **G-amide**

Bold indicates the amino acids of the shared pentapeptide amide C-terminal region

Table 2.3. The amino acid sequences of the CRF-like diuretic peptides

MasDH	RM**PSLSI**DL**P**MS**VLRQ**K**LSLE**KE**R**K VHALR**AAANRNFLN**DI-**NH2**
MasDPII	SFSVNPAVDILQHRYMEKV**AQ**N**NR**N**FLN**RV-**NH2**
Locusta	MGMG**PSLSIVNP**M**DVLRQRLLLEIARRR**LRDAEE **QI**K**AN**K**DFL**QQ**I-NH2**
Acheta	TGAQ**SLSIVAPLDVLRQRL**MN**E**LN**RRRMRE**LQGSR**IQQNR**QL**LTS**I-NH2**
Periplnt	TGSG**PSLSIVNPLDVLRQRLLLEIARRRMR**QSQD **QIQANRE**I**L**QT**I-NH2**
Msca/Stx	N**KPSLSIVNPLDVLRQRLLLEIARRQMKE**NTR **Q**VEL**NR**AI**L**KNV-**NH2**
Culex	T**KPSLSIVNPLDVLRQR**IIL**E**M**ARRQMRE**NTR **Q**VER**NK**AI**L**RE**I-NH2**
Tenebrio	TS**P**TI**SI**TA**P**I**DVLR**KTWEQE**R**A**RKQM**VK **NRE**F**LN**SLN-OH
Hum-CRF	SEE**P**PI**S**LDLTFHLLRE V**LE**MARAEQLA **QQ**AHS**NR**KLMEI**I-NH2**

Abbreviations: MasDH; *M. sexta* diuretic hormone, MasDPII; *M. sexta* diuretic peptide II, Periplnt; *Periplaneta*, Msca/Stx; *Musca/Stomoxys*, hum-CRF; human CRF
Bold indicates amino acid similarities with three or more sequences

RESULTS

Urine production and excretion in control groups

Initial experiments were conducted to measure differences in urine production in blood fed intact, blood fed decapitated and non blood fed mosquitoes following injection of saline-diluted radiolabeled water. Urine production for blood fed intact mosquitoes reached a maximum 30 minutes following injection (Figure 2.1). Blood fed decapitated mosquitoes had significantly lower urine production than blood fed intact mosquitoes ($p<0.001$) (Figure 2.1). Urine production values did not exceed the 60 minute value throughout the incubation period for intact non blood fed, injected mosquitoes.

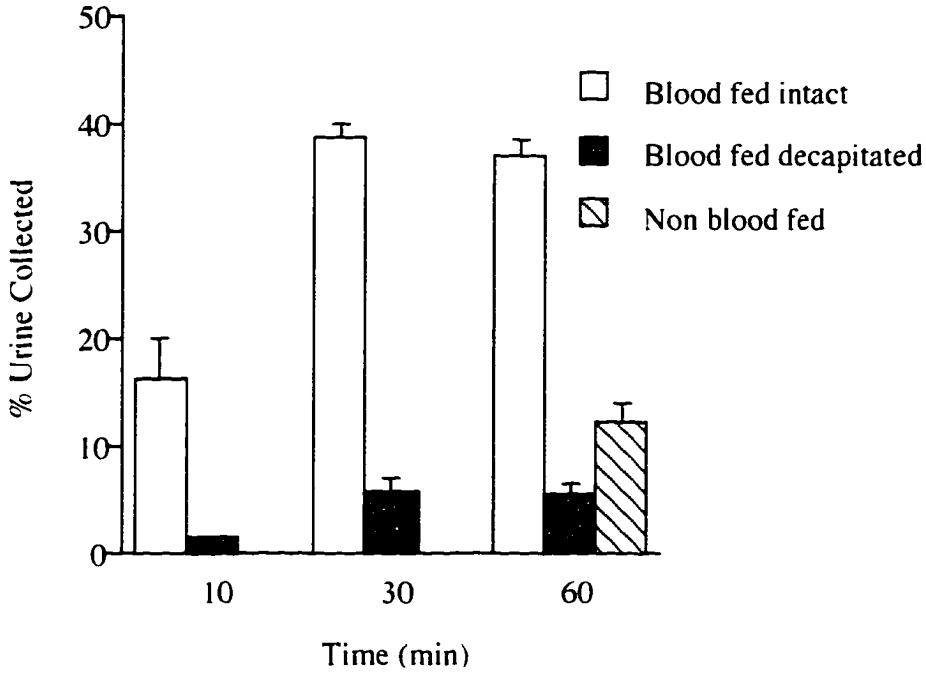

Fig. 2.1. In vivo urine production bioassay comparing blood fed intact, blood fed decapitated and non blood fed intact mosquitoes. Values are means ± SE, 10 mosquitoes per bar, when not shown the error bars were too small to appear on the graph.

The effect of 5-Hydroxytryptamine (5-HT) on urine production

To determine if in vivo urine production is influenced by 5-HT, injection dose response experiments were performed using the in vivo urine production bioassay. A dose response curve for urine production stimulated by 5-HT is presented in Figure 2.2A. The ED_{50} calculated value was 4.20×10^{-5} μmoles based on the points making up the linear

increase in urine production for the line from the control to the maximum point of % urine collected.

The effect of putative diuretic peptides on in vivo urine production

Dose response curves showing urine production stimulated by the CDPs are presented in Figures 2.2B,C,D. ED_{50} values based on dose response curves were 8.55 X 10^{-4} nmoles for CDP-I, 1.92 X 10^{-4} nmoles for CDP-II and 3.65 X 10^{-4} nmoles for CDP-III.

Dose response curves showing the effect of ALPs on urine production are presented in Figures 2.3A,B,C. The ED_{50} values for ALP-I, ALP-II and ALP-III are 1.53 X 10^{-4}, 3.56 X 10^{-4} and 3.70 X 10^{-5} nmoles respectively.

Figure 2.3D shows the dose response curve for the *C. salinarius* diuresin peptide stimulated increases in urine production. Diuresin stimulated dose curve resulted in an ED_{50} value of 6.82 X 10^{-4} nmoles which was similar to many of the mosquito leucokinin values.

Urine production in the dose response curves is expressed as % urine collected which allows for comparison between different agonist stimulated dose response curves obtained for different agonists (Table 2.4). The ED_{50} value of 4.20 X 10^{-2} nmoles for 5-HT represented the least potent of the agonists tested in our bioassay. ALP-III was the most potent mosquito leucokinin tested with an ED_{50} of 1.53 X 10^{-5} nmoles. CDP-II was the most potent of the culekinin depolarizing peptides with an ED_{50} of 1.92 X 10^{-4} nmoles. The *C. salinarius* diuresin dose curve was similar to the leucokinin stimulated dose curves and resulted in an estimated ED_{50} of 3.1 X 10^{-4} nmoles. This was surprising because in other insects the diuresin have been found to be more potent diuretic agonists then the leucokinins.

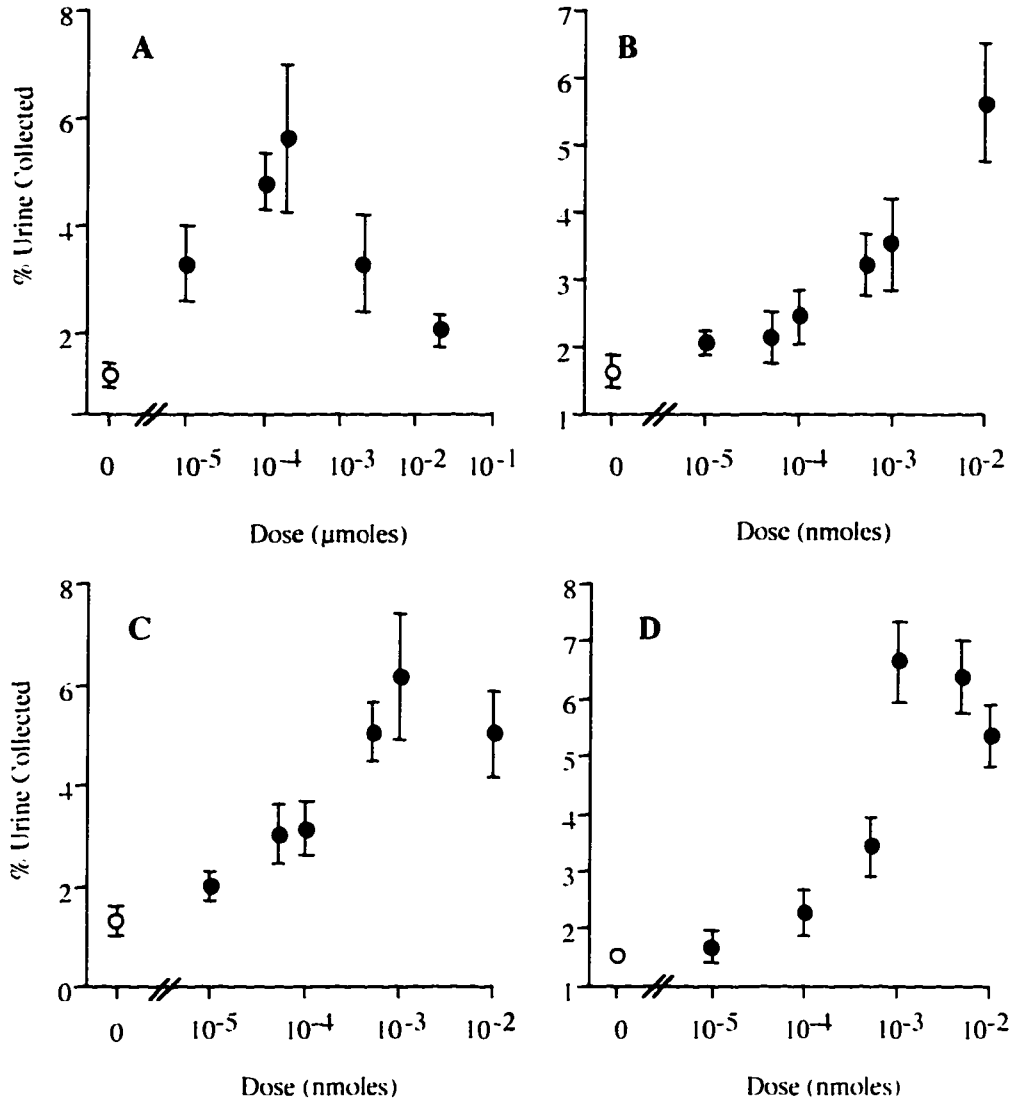

Fig. 2.2. Dose-response curves showing the effect of agonists on in vivo urine production in mosquitoes which were blood fed and decapitated: (A) 5-hydroxytryptamine (5-HT); (B) culekinin depolarizing peptide I (CDP-I); (C) culekinin depolarizing peptide II (CDP-II); (D) culekinin depolarizing peptide III (CDP-III). Open symbols represent control mosquitoes (without agonist), values represent means ± SE, n = 8 mosquitoes per point, when not shown the error bars were too small to appear on the graph.

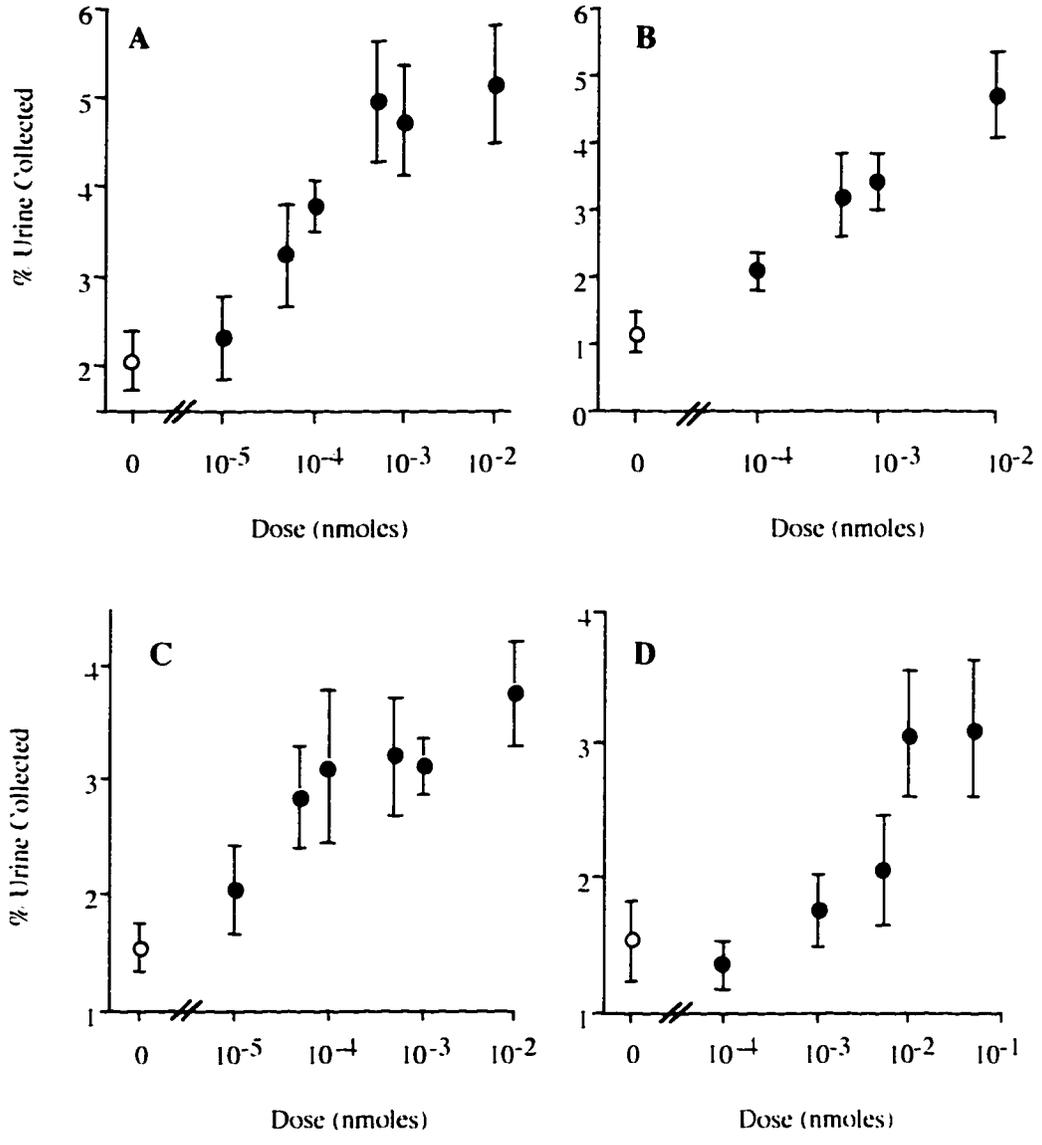

Fig 2.3. Dose-response curves showing the effect of agonists on in vivo urine production in mosquitoes which were blood fed and decapitated: (A) *A. aegypti* leucokinin peptide I (ALP-I); (B) *A. aegypti* leucokinin peptide II (ALP-II); (C) *A. aegypti* leucokinin peptide III (ALP-III); (D) *C. salinarius* diuresin. Open symbols represent control mosquitoes (without agonist), values represent means ± SE, n = 8.

Table 2.4. Data from different agonist stimulated dose response curves.

Agonist	Y-intercept dose curve (linear fit)	Slope dose curve (linear fit)	Maximum urine collected (fold above zero)	ED50 (nmoles)
5-HT	2.03	18.38	4.5	4.20×10^{-2}
CDP-I	2.55	0.31	3.5	8.55×10^{-4}
CDP-II	2.25	4.28	4.7	1.92×10^{-4}
CDP-III	1.55	4.82	4.3	3.65×10^{-4}
ALP-I	2.53	0.26	2.5	1.53×10^{-4}
ALP-II	2.55	0.28	4.5	3.56×10^{-4}
ALP-III	2.24	0.25	3.1	3.70×10^{-5}
diuresin	1.45	0.15	2.0	6.82×10^{-4}

Control mosquitoes were injected without agonist
8 mosquitoes per dose point with 40 - 56 mosquitoes per curve
Means are ± SEM

Immunoneutralization experiments using antibodies against peptide agonists

Immunoneutralization experiments were performed to determine if injecting mosquitoes with antibodies raised against either the *C. salinarius* diuresin or ALP-I would inhibit peptide stimulated increases in urine production.

Table 2.5. Effects of antibody injection on peptide stimulated urine production.

Treatment	Urine Collected (% ± SEM)	p
Baseline	2.1 ± 0.6	
NRS	8.1 ± 1.3	
Anti-CRF DP	5.8 ± 0.6	<0.05
Baseline	0.9 ± 0.1	
NRS	6.6 ± 0.8	
Anti ALP-I	3.8 ± 0.4	<0.01

Baseline mosquitoes were injected with the appropriate antibody and given a second injection without peptide
Baseline group consisted of from 4 - 6 mosquitoes,
NRS/antibody injected consisted of from 12 - 15 mosquitoes.
Values of p were determined by the statistical application of the students t-test for NRS versus Antibody injected

Mosquitoes were injected with antibody, blood fed, decapitated and injected 16 - 18 hours later with either the *C. salinarius* diuresin or ALP-I peptide. Urine production was measured and statistically compared in mosquitoes injected with or without antibody.

Table 2.5 represents the results from the immunoneutralization experiments using polyclonal antibodies raised against either the *C. salinarius* diuresin or ALP-I. Injecting antibodies against either the diuresin or ALP-I significantly inhibited the response to peptides compared to mosquitoes injected with saline. Mosquitoes injected with antibody did not, however, demonstrate a complete block of urine production as levels remained above urine production in controls.

DISCUSSION

The in vivo urine production bioassay was demonstrated to be a valid method to test the effect of agonists on blood fed mosquitoes. Recovery of radiolabeled water from injected mosquitoes and collected urine averaged 98.1%. It was demonstrated that decapitation effectively removed the source(s) of endogenous diuretic endocrine factor(s) (Wheelock et al., 1988). Increased urine production following the injection of agonist likely represents the stimulation due to the agonist and not the effects of endogenous endocrine factors.

5-HT was chosen as the initial agonist for dose response stimulation experiments because it is biologically active in several insect groups. 5-HT has been shown to function as a diuretic hormone in the blood feeding insect *Rhodnius prolixus* (Maddrell et al., 1969; Lange et al., 1989; Maddrell et al., 1991; Maddrell et al., 1993) and in larval *A. aegypti* (Clark, 1994; Clark and Bradley, 1996).

5-HT stimulated in vivo urine production in mosquitoes in a dose-dependent manner. The ED_{50} for 5-HT in urine production experiments was nearly 100 fold higher than other diuretic agonists. Doses greater than 2×10^{-4} μmoles inhibited urine production, suggesting either the occurrence of receptor down regulation, or the possibility that more than one type of 5-HT receptor may be active in the mosquito. Multiple subtypes of the 5-HT receptor have been recognized in *Drosophila melanogaster* (Saudou et al., 1992). Receptors for 5-HT have been characterized in the tubules of larval *A. aegypti* (Clark and Bradley, 1997). Alternatively, 5-HT injections above 2×10^{-4} μmoles could

influence either the midgut or the rectum. Higher doses of 5-HT could enhance solute and water movement across the midgut resulting a dilution of 5-HT titers in the hemolymph, preventing 5-HT stimulation of the Malpighian tubules. Increased doses of 5-HT could stimulate rectal absorption resulting in an increase in solute and fluid transport from the rectal lumen into the hemolymph. Stimulation of either the midgut or the rectum with higher doses of 5-HT would likely result in lower total urine production, providing a possible explanation for reduced urine volumes at higher 5-HT doses. In the present study, extremely high doses of 5-HT were required to elicit detectable increases in urine production, further complicating the assessment of the role of 5-HT in tubule function. Stimulation of urine production in a dose-dependent manner indicates biological activity and suggests 5-HT may have a role in modulating tubule function, however, it is questionable that 5-HT has a significant role in the regulation of urine production in the adult mosquito. The role of 5-HT in the physiology of the adult mosquito will remain uncertain until additional immunohistochemical data is provided and changes in 5-HT hemolymph concentrations can be correlated with feeding status in the adult mosquito.

In our bioassay, the *C. salinarius* diuresin was biologically active, stimulating in vivo urine production in a dose dependent manner. However, urine production and ED_{50} values were not as impressive as those following treatment with leucokinins. The diuresins have been shown to be potent stimulators of tubule fluid secretion and intracellular cAMP in insects (Coast et al., 1992; Thompson et al., 1995; Audsley et al., 1995). The *C. salinarius* diuresin is a 44 amino acid peptide with two methionines at positions 22 and 27. Oxidation of methionine residues during storage decreases biological activity (Coast et al., 1992; Audsley et al., 1995) and may also account for the low response observed in our bioassay. *C. salinarius* diuresin analogs, which have fewer reactive groups (such as those that have norleucine in place of methionine), could be used in our urine production bioassay to assess whether the loss of biological activity was due to peptide degradation.

The low bioactivity of the *C. salinarius* diuresin may have been due to reduced solubility of the peptide following reconstitution with *Aedes* saline. The *C. salinarius* diuresin was stored dry, with bovine serum albumin to prevent adsorption, and reconstituted with *Aedes* saline prior to use in the bioassays. Coast and Kay (1994), and Patel et al. (1995), have demonstrated that reconstituting dried *Acheta* diuresin in methanol may dissolve the peptide more efficiently. We chose to use *Aedes* saline to prepare peptides for bioassays because dilute methanol might interfere with normal tubule function.

The mosquito leucokinins stimulated increases in in vivo urine production in a dose-dependent manner. Based on the dose response curve, mosquito leucokinins were ranked according to the estimated ED50 values, from high to low, CDP-I > CDP-III > ALP-II > CDP-II > ALP-I > ALP-III. Although differences between ED50 values for the leucokinins were not statistically significant, ranking peptides in this manner may suggest a trend in agonist/receptor binding affinity (Coast et al., 1990). Treatment of mosquitoes with CDP-I, demonstrated relatively low receptor binding affinity, as evidenced by the highest ED50. CDP-I and ALP-II share amino acid sequence similarities, but vary at position five of the C-terminal pentapeptide core sequence. Differences in CDP-I at a single amino acid residue could possibly reduce recognition and binding to the *A. aegypti* receptor because the C-terminal pentapeptide region is important in receptor recognition (Hayes et al., 1989). It is not surprising that the receptor binding affinity would be higher for ALP-II, which represents the endogenous peptide for *A. aegypti*. The *A. aegypti* leucokinin peptides were at the lower end of the ED50 ranking range, suggesting that the endogenous peptides tended to have higher receptor binding affinities, compared to the culekinins. This is more apparent with ALP-III which had a 10 fold lower ED50 compared to the other mosquito leucokinins tested.

Immunization of mosquitoes with polyclonal antisera raised against either ALP-I or *C. salinarius* diuresin resulted in significant inhibition of peptide stimulated urine production. Inhibition of urine production by test peptide in mosquitoes treated with antibody indicates that the antisera remained capable of binding peptide throughout the incubation period. In the immunoneutralization experiments mosquitoes were given a second injection containing test peptide 15-18 hours following injection with the antisera. If the injected antisera remained active during our experiments then it is likely that the inhibition of stimulated urine production was due to antisera binding to and inactivating the injected peptide. Patel et al. (1995), demonstrated that injected polyclonal antisera can remain active in vivo in insects for at least 12 hours. These data are not surprising as Tublitz and Evans (1986), used a monoclonal antibody to block the actions of cardioactive neuropeptide in *M. sexta*. The incomplete inhibition, not to baseline values, of stimulated urine production in mosquitoes treated with antibody may have been due to the partial degradation of the antibody or to sub optimal titers of antibody. The inhibition of urine production following antibody treatment suggests that ALP-I and *C. salinarius* diuresin directly stimulate urine production in the mosquito.

Biological activity of the mosquito leucokinins has been suggested based on in vitro methods that measure changes in tubule transepithelial voltage, hindgut myotropic activity and increases in primary urine secretion (Hayes et al., 1994; Veenstra et al., in press). These methods are rapid and sensitive but do not measure changes in total urine production. In this study, we report a dose dependent in vivo stimulation of total urine production by the mosquito leucokinins. Additionally, significant inhibition of peptide-stimulated urine production occurred following immunization with ALP-I antisera. Others have reported the isolation of a cDNA encoding the prepro-form of *A. aegypti* leucokinins (Veenstra et al., in press) and immunolocalization of leucokinin reactive neurosecretory cells (Chen et al., 1994b). Based on these data we suggest that the mosquito leucokinins are biologically active and are potentially important agonists in the regulation of urine production in the mosquito.

The evidence presented above suggests that mosquito leucokinins are probably important in the neuroendocrine regulation of urine production in the mosquito. To confirm this it will be necessary to measure these peptides in the hemolymph, during specific times of the adult life cycle. Patel et al. (1994), were able to measure levels of neuropeptide diuretic factor in the hemolymph of the locust. With the availability of antibodies against the mosquito leucokinins, measurement of leucokinin concentrations in the hemolymph of mosquitoes will soon be possible.

Acknowledgments--We thank Julia Guzova for her technical assistance. We gratefully acknowledge Dr. Jan Veenstra for providing the *Aedes* leucokinin peptides, the ALP-I and *C. salinarius* diuresin antibodies and for his comments and suggestions and Dr. Timothy Hayes for providing the culekinin depolarizing peptides. This work was supported in part by NSF grant number DCB-8918437, MacArthur Foundation Vector Biology Grant to the Center for Insect Science and the Agricultural Experiment Station at the University of Arizona.

REFERENCES

Audsley N., Coast G. M. and Schooley D. A. (1993) The effects of *Manduca sexta* diuretic hormone on fluid transport by the Malpighian tubules and cryptonephric complex on *Manduca sexta*. *J. exp. Biol.* **178**, 231-243.

Audsley N., Kay I., Hayes T. K. and Coast G. M. (1995) Cros reactivity studies of CRF-related peptides on insect Malpighian tubules. *Comp. Biochem. Physiol.* **110A**, 87-93.

Blackburn M. B., Wagner R. M., Shabanowitz J., Kochansky J. P., Hunt D. F. and Raina A. K. (1995) The isolation and identification of three diuretic kinins from the abdominal ventral nerve cord of adult Helicoverpa zea. *J. Insect Physiol.* **41**, 723-730.

Blackburn M. B., Kingan T. G., Bodnar W., Shabanowitz J., Hunt J., Kempe T., Wagner R. M., Raina A. K., Schnee M. E. and Ma M. C. (1991) Isolation and identification of a new diuretic peptide from the tobacco hornworm, *Manduca sexta*. *Biochem. Biophys. Res. Comm.*, **181**, 927-932.

Bradley T. J. (1985) The excretory system: structure and function. In: *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, (Ed. Kerkut G. A. and Gilbert L. I.), pp 421-465. Pergamon, London/New York.

Cantera R., Hansson B. S., Hallberg E. and Nassel D. R. (1992) Postembryonic development of leucokinin I-immunoreactive neurons innervating a neurohemal organ in the turnip moth *Agrotis segetum*. *Cell Tissue Res.* **269**, 65-77.

Chen Y., Veenstra J. A., Davis N. T. and Hagedorn H. H. (1994a) A comparative study of leucokinin-immunoreactive neurons in insects. *Cell Tissue Res.* **276**, 69-83.

Chen Y., Veenstra J. A., Hagedorn H. and Davis N. T. (1994b) Leucokinin and diuretic hormone immunoreactivity of neurons in the tobacco hornworm, Manduca sexta, and co-localization of this immunoreactivity in lateral neurosecretory cells of abdominal ganglia. *Cell Tissue Res.* **278**, 493-507.

Clark T. M. (1994) Ph.D. Dissertation, University of California, Irvine. Hormonal control of Malpighian tubules in larval *Aedes aegypti*.

Clark T. M. and Bradley T. J. (1996) Stimulation of Malpighian tubules from larval *Aedes aegypti* by secretagogues. *J. Insect Physiol.* **42**, 593-602.

Clark T. M. and Bradley T. J. (1997) Malpighian tubules of larval *Aedes aegypti* are hormonally stimulated by 5-HT in response to increased salinity. *Arch. Insect Biochem. and Physiol.* **34**, 123-141.

Clottens F. L., Holman M. G., Coast G. M., Totty N. F., Hayes T. K., Kay I., Mallet A. I., Wright M. S., Chung J. S., Truong O. and Bull D. L. (1994) Isolation and characterization of a diuretic peptide common to the house fly and stable fly. *Peptides.* **15**, 971-979.

Coast G. M. and Kay I. (1994) The effects of *Acheta* diuretic peptide on isolated Malpighian tubules from the house cricket *Acheta domesticus*. *J. Exp. Biol.* **187**, 225-243.

Coast G. M., Holman G. M. and Nachman R. J. (1990) Diuretic activity of a series of cephalomyotropic neuropeptides, the achtakinins, on isolated Malpighian tubules of the house cricket (*Acheta domesticus*). *J. Insect Physiol.* **36**, 481-488.

Coast G. M., Hayes T. K., Kay I. and Chung J. (1992) Effect of *Manduca sexta* diuretic hormone and related peptides on isolated Malpighian tubules of the house cricket *Aceta domesticus* (L). J. exp. Biol. **162**, 331-338.

Digan M. E., Roberts D. N., Enderlin F. E., Woodworth A. R. and Kramer S. J. (1992) Characterization of the precursor for *Manduca sexta* diuretic hormone Mas-DH. *Proc. Natl. Acad. Sci.* **89**, 11074-11078.

Furuya K., Schegg K. M., Wang H., King D. D., and Schooley D. A. (1995) Isolation and identification of a diuretic hormone from the mealworm *Tenebrio molitor.* Proc. Natl. Acad. Sci. USA **92**, 12323-12327.

Hagedorn H. H., Turner S., Hagedorn E. A., Pontecorvo D., Greenbaum P., Pfeiffer D., Wheelock G. and Flanagan T. R. (1977) Postemergence growth of the ovarian follicles of *Aedes aegypti. J. Insect Physiol.* **23**, 203-206.

Hayes T. K., Pannabecker T. Hinckley D. Holman G. Nachman R. Petzel D. and Beyenbach K. W. (1989) Leucokinins, a new family of ion transport stimulators and inhibitors in insect Malpighian tubules. *Life Sci.* **44**, 1259-1266.

Hayes T. K., Holman G. M. Pannabecker T. L. Wright M. S. Strey A. A. Nachman R. J. Hoel D. F. Olson J. K. and Beyenbach K.W. (1994) Culekinin depolarizing peptide: a mosquito leucokinin-like peptide that influences insect Malpighian tubule ion transport. *Regul. Pept.* **52**, 235-248.

Hegarty J. L., Zhang B. Pannabecker T. L. Petzel D. H. Baustian M. D. and Beyenbach K. W. (1991) Dibutyryl cAMP activates bumetanide-sensitive electrolyte transport in Malpighian tubules. *Am. J. Physiol.* **261**, C521-C529.

Holman G. M., Cook B. J. and Nachman R. J. (1986a) Isolation, primary structure and synthesis of two neuropeptides from *Leucophaea maderae:* members of a new family of cephalomyotropins. *Comp. Biochem. Physiol.* **84C**, 205-211.

Holman G. M., Cook B. J. and Nachman R. J. (1986b) Isolation, primary structure and synthesis of two additional neuropeptides from *Leucophaea maderae:* members of a new family of cephalomyotropins. *Comp. Biochem. Physiol.* **84C**, 271-276.

Holman G. M., Cook B. J. and Nachman R. J. (1987a) Isolation, primary structure and synthesis of leucokinins V and VI. *Comp. Biochem. Physiol.* **88C**, 27-30.

Holman G. M., Cook B. J. and Nachman R. J. (1987b) Isolation, primary structure and synthesis of leucokinins VII and VIII: the final members of this new family of cephalomyotropins from head extracts of *Leucophaea maderae. Comp. Biochem. Physiol.* **88C**, 31-34.

Holman G. M., Nachman R. J. and Wright M. S. (1990) Comparative aspects of insect myotropic peptides. *Prog. Clin. Biol. Res.* **342**, 35-39.

Kataoka H., Troetschler R. G. Li J. P. Kramer S. J. Carney R. L. and Schooley D. A. (1989) Isolation and identification of a diuretic hormone from the tobacco hornworm, *Manduca sexta. Proc. Natl. Acad. Sci.* **86**, 2976-2980.

Kay I., Coast G. M. Cusinato O. Wheeler C. H. Totty N. F. and Goldsworthy G. J. (1991a) Isolation and characterization of a diuretic peptide from *Acheta domesticus*, evidence for a family of insect diuretic peptides. *Biol. Chem. Hoppe-Seyler.* **372**, 505-512.

Kay I., Patel M. Coast G. M. Totty N. F. Mallet A. I. and Goldsworthy G. J. (1992) Isolation, characterization and biological activity of a CRF-related diuretic peptide from *Periplaneta americana* L. *Regul Pept.* **42**, 111-122.

Kay I., Wheeler C. H. Coast G. M. Totty N. F. Cusinato O. Patel M. and Goldsworthy G. J. (1991b) Characterization of a diuretic peptide from *Locusta migratoria. Biol. Chem. Hoppe-Seyler.* **372**, 929-934.

Lange A. B., Orchard I. and Barrett F. M. (1989) Changes in haemolymph serotonin levels associated with feeding in the blood-sucking bug, *Rhodnius prolixus. J. Insect Physiol.* **35**, 393-399.

Lehmberg E., Ota R., Furuya K., King D., Applebaum S., Ferenz H. and Schooley D. (1991) Indentification of a diuretic hormone of *Locusta migratoria. Biochem. Biophys. Res. Commun.* **179**, 1036-1041.

Maddrell S. H. P., Pilcher D. E. M. and Gardiner, B. O. C. (1969) Stimulatory effects of 5-hydroxytryptamine (serotonin) on secretion of the Malpighian tubules of insects. *Nature* **222**, 784-785.

Maddrell S. H. P., Herman W. S. Farndale R. W Riegel J. A. (1993) Synergism of hormones controlling epithelial fluid transport in an insect. *J. Exp. Biol.* **174**, 65-80.

Maddrell S. H. P., Herman W. S. Mooney R. L. and Overton J. A. (1991) 5-Hydroxytryptamine: A second diuretic hormone in *Rhodnius prolixus*. *J. exp. Biol.* **156**, 557-566.

Nassel D. R., Cantera R. and Karlsson A. (1992) Neurons in the cockroach nervous system reacting with antisera to the neuropeptide leucokinin I. *J Comp Neurol* **322**, 45-67.

Pannabecker T. (1995) Physiology of the Malpighian tubule. *Ann. Rev. Entomol.* **40**, 493-510.

Pannabecker T. L., Hayes T. K. and Beyenbach K. W. (1993) Regulation of epithelial shunt conductance by the peptide leucokinin. *J. Membr. Biol.* **132**, 63-76.

Patel M., Hayes T. K. and Coast G. M. (1995) Evidence for the hormonal function of a CRF-related diuretic peptide (Locusta-DP) in *Locusta Migratoria*. *J. Exp. Biol.* **198**, 793-804.

Patel M., Chung J. S., Kay I., Mallet A. I. Gibbon C. R. Thompson K. S. J. Bacon J. P. and Coast G. M. (1994) Localization of *Locusta*-DP in Locust CNS and hemolymph satisfies initial hormonal criteria. *Peptides*. **15**, 591-602.

Petzel D. H., Hagedorn H. H. and Beyenbach K. W. (1985) Peptide nature of two mosquito natriuretic factors. *Am. J. Physiol.* **250**, R328-R332.

Petzel D. H., Hagedorn, H. H. and Beyenbach K. W. (1986) Preliminary isolation of mosquito natriuretic factor. *Am. J. Physiol.* **249**, R379-R386.

Saudou F., Boschert U. Amlaiky N., Plassat J. L. and Hen R. (1992) A family of *Drosophila* serotonin receptors with distinct intracellular signalling properties and expression patterns. *EMBO. J.* **11**, 7-17.

Schoofs L., Holman G. M., Proost P., Damme J. V., Hayes T. K. and Loof A. D. (1992) Locustakinin, a novel myotropic peptide from *Locusta migratoria*, isolation, primary structure and synthesis. *Regul. Pept.* **37**, 49-57.

Shapiro, J. P. and Hagedorn, H. H. (1982) Juvenile hormone and the development of ovarian responsiveness to a brain hormone in the mosquito, *Aedes aegypti*. *Gen. Comp. Endocrinol.* **46**, 176-183.

Thompson K. S. J., Rayne R. C., Gibbon C. R., May S. T., Patel, M., Coast G. M. and Bacon J. P. (1995) Cellular colocalization of diuretic peptides in locusts: a potent control mechanism. *Peptides*. **16**, 95-104.

Tublitz N. J. and Evans P. D. (1986) Insect cardioactive peptides: cardioacceleratory peptide (CAP) activity is blocked in vivo and in vitro with a monoclonal antibody. *J Neurosci*. **6**, 2451-2456.

Veenstra J. A. (1988) Effects of 5-hydroxytryptamine on the Malpighian tubules of *Aedes aegypti*. *J. Insect Physiol*. **34**, 299-304.

Veenstra J. A. (1994) Isolation and identification of three leucokinins from the mosquito *Aedes aegypti*. *Biochem. Biophys. Res. Comm*. **202**, 715-719.

Veenstra J. A. and Hagedorn H. H. (1991) Indentification of neuroendocrine cells producing a diuretic hormone in the tobacco hornworm moth, *Manduca sexta*. *Cell Tissue Res*. **266**, 359-364.

Wheelock G. H., Petzel D. H., Gillett J. D., Beyenbach K. W. and Hagedorn H. H. (1988) Evidence for hormonal control of diuresis after a blood meal in the mosquito *Aedes aegypti*. Arch. *Insect Biochem. Physiol*. **7**, 75-89.

Wheelock G. H. Sieber K. P. and Hagedorn H. H. (1991) Rapid isolation of a neurohormone from mosquito heads by high-performance liquid chromatography. *J. Chromatog*. **542**, 508-514.

Williams J. C. and Beyenbach K. W. (1983) Differential effects of secretagogues on Na and K secretion in the Malpighian tubules of *Aedes aegypti*. *J. Comp. Physiol*. **149**, 511-517.

Williams J. C., Hagedorn H. H. and Beyenbach K. W. (1983) Dynamic changes in flow rate and composition of urine during the post-blood meal diuresis in *Aedes aegypti*. *J. Comp. Physiol*. **153**, 257-265.

3. EFFECTS OF PUTATIVE DIURETIC FACTORS ON INTRACELLULAR SECOND MESSENGER LEVELS IN THE MALPIGHIAN TUBULES OF *AEDES AEGYPTI*

Craig Cady and Henry H. Hagedorn
The Center for Insect Science of the University of Arizona, Tucson, Arizona, USA
The Department of Entomology, 410 Forbes Bldg. 36, University of Arizona, Tucson, Arizona, USA

Abstract--Intracellular levels of the second messengers, 3',5'-cyclic adenosine monophosphate (cAMP) and inositol 1,4,5-trisphosphate (IP_3) were measured in cells from the Malpighian tubules of *Aedes aegypti* following the application of 5-hydroxytryptamine (5-HT) and the putative mosquito diuretic peptides, *Culex salinarius* diuresin and mosquito leucokinins (CDP-I, II, III, ALP-I, II and III) in vitro. The *C. salinarius* diuresin significantly ($p< 0.05$) increased tubule intracellular cAMP concentrations. Treatment of tubules with either 5-HT or CDP-II resulted in significant increases in both intracellular cAMP and IP_3 concentrations. All of the mosquito leucokinins, with the exception of CDP-I, significantly stimulated intracellular IP_3 in isolated tubules. Data presented here suggest that the mosquito leucokinins may function on the Malpighian tubules of *A. aegypti* by increasing the intracellular Ca^{2+} levels through the release of IP_3 sensitive Ca^{2+} stores. The physiological relevance of these data to the regulation of mosquito Malpighian tubule function is discussed.

INTRODUCTION

Most terrestrial insects, have a large surface to volume ratio and must compensate for large evaporative water losses by conserving water (Phillips, 1981). Because of this, the rapid excretion of urine (diuresis) is not common among the insects. However, at selected times in the life cycle of some insects, a diuresis is necessary. The haematophagous yellow fever mosquito, *Aedes aegypti* is able to ingest large meals of up to 10 times their body volume (Williams and Beyenbach, 1983). Water, solute and nutrients from the blood meal are absorbed across the midgut epithelia producing changes in the ionic strength and composition of the hemolymph. In the yellow fever mosquito, *A. aegypti*, the post blood

meal diuresis involves movement of ions and water from the midgut to the Malpighian tubules via the hemolymph. Diuresis after the blood meal can reach a peak rate of about 5 urine droplets/minute (50 nl/min) resulting in a loss of 40% of the ingested plasma volume within the first hour after the blood meal (Williams and Beyenbach, 1983).

The post blood meal diuresis in the mosquito may be regulated by endocrine factors (Wheelock et al., 1988). In insects, two families of neuropeptides have been found to stimulate fluid secretion in Malpighian tubules, the diuresins or the corticotropin-releasing factor-like diuretic hormones (CRF-like DH) and the leucokinins, originally isolated from *Leucophaea maderae* (Holman et al., 1986a, b; 1987a, b).

The diuresins, share amino acid sequence similarities to the corticotropin-releasing factor (CRF-like diuretic hormones) family of peptides. Initially isolated from *Manduca sexta* (Kataoka et al., 1989; Blackburn, 1991), the diuresins have been isolated from several insect species including *Acheta domesticus* (Kay et al., 1991a), *Locusta migratoria* (Lehmberg, et al., 1991; Kay et al., 1991b), *Periplaneta americana* (Kay et al., 1992), one identical peptide from *Musca domestica* and *Stomoxys calcitrans* (Clottens et al., 1994) and *Tenebrio molitor* (Furuya et al., 1995). *M. sexta* diuresin immunoreactive cells have been identified in neurosecretory cell axon terminals in the corpus cardiacum of *M. sexta* (Veenstra and Hagedorn, 1991; Blackburn, 1991). In *Locusta migratoria*, a diuresin has been identified in the corpus cardiacum, abdominal ganglia and in the hemolymph (Patel et al., 1994; Thompson et al., 1995). A cDNA for the CRF-like diuretic hormones has been isolated in *M. sexta* and was found to encode for the precursor form of this peptide (Digan et al., 1992).

Diuresins increase fluid secretion in Malpighian tubules via a 3',5'-cyclic adenosine monophosphate (cAMP) dependent mechanism (Kay et al., 1991a, b; Kay et al., 1992; Lehmberg et al., 1991; Audsley et al., 1993). Recently, a putative mosquito diuretic hormone has been purified and sequenced from *C. salinarius* by Dr. F. Clottens of the U.S. Department of Agriculture (Clottens, personal communication) and synthesized by the Division of Biotechnology of the University of Arizona. The amino acid sequence of this *C. salinarius* peptide closely resembles the diuresin family of neuropeptides.

The leucokinins are a group of myotropic octapeptides which share a bioactive pentapeptide amide C-terminal region (Hayes et al., 1989; Hayes et al., 1994). Leucokinins have been isolated and sequenced from locusts (Schoofs et al., 1992), crickets (Holman, 1990) and moths (Blackburn et al., 1995). Leucokinins have been isolated from two species of mosquitoes *C. salinarius*, the culekinin depolarizing peptides (CDP) (Hayes et al., 1994)

and *A. aegypti*, leucokinin peptides (ALP) (Veenstra, 1994). To date, leucokinin immunoreactive neurosecretory cells have been identified from *M. sexta*, *Acheta domesticus*, *Schistocerca americana*, *Apis mellifera*, *Agrotis segetum*, *Leucophaea maderae* and *A. aegypti* (Chen et al., 1994a; Chen et al., 1994b; Thompson et al., 1995; Cantera et al., 1992; Nassel et al., 1992). Only recently has a cDNA been isolated from *A. aegypti* and was found to encode for the preproleucokinin, indicating that the three *A. aegypti* leucokinins are represented by a single gene (Veenstra et al., in press).

In isolated Malpighian tubules, leucokinins increase the secretion of electrolytes (NaCl and KCl) and water in *A. aegypti* by increasing Cl^- conductance across the tubule via an undetermined paracellular pathway (Hayes et al., 1989; Pannabecker et al., 1993). As is the case with other neuropeptides, this process is likely receptor mediated (Pannabecker et al., 1993; Hayes, personal communication). The only reported evidence for diuretic hormone receptors in insect Malpighian tubules is the identification of a cDNA which encodes a CRF-related diuretic hormone receptor in *M. sexta* (Reagan, 1994).

Many neuropeptides, including the diuresins, are thought to regulate epithelial cell function via cAMP dependent mechanisms. However, the intracellular signaling mechanism for the leucokinins is not well understood. Previous work with tubule stimulating factors suggests that cAMP independent mechanisms are important. In locusts, two distinct diuretic factors were isolated from corpora cardiaca extracts; only one of which stimulated tubule fluid secretion via a cAMP dependent mechanism (Morgan and Mordue, 1985). Exposure of isolated tubules to corpora cardiaca extracts stimulated both cAMP and inositol 1,4,5-trisphosphate (IP_3) in locusts (Fogg et al., 1990). Achetakinins (leucokinins isolated from the cricket) increased tubule fluid secretion independently of cAMP, probably via changes in cellular Ca^{2+} (Coast et al., 1990; Coast et al., 1993). In *Drosophila melanogaster*, leucokinin-stimulated increases in tubule secretion were independent of both cAMP and cGMP. The increase in tubule secretion was similar to that exhibited by tubules stimulated, with the Ca^{2+} mobilizing drug thapsigargin (Davies et al., 1995). It was later determined in *D. melanogaster* that leucokinin mediated changes in tubule Cl^- conductance were indeed dependent on cellular Ca^{+2} and distinct from cation transport processes which appear to be regulated by cyclic nucleotides (O'Donnell et al., 1996). The mosquito leucokinins have been shown to cause depolarization of the transepithelial voltage and stimulate fluid secretion (Hayes et al., 1994; Veenstra, personal communication). It is therefore reasonable to assume that these physiological effects on the Malpighian tubules are mediated through the hydrolysis

of phosphatidylinositol leading to the production of the cellular second messenger IP_3 which is associated with increases in cellular Ca^{2+}.

The mosquito diuresin and mosquito leucokinins bear close amino acid sequence homology to the two major neuropeptide families implicated in regulating fluid secretion at the Malpighian tubules, the CRF-like diuretic peptides and myotropic leucokinins. Previous data suggests that these families mediate their action at the Malpighian tubules via different intracellular signal transduction pathways. The diuresins appear to function via a receptor mediate activation of adenylate cyclase leading to an increase in cellular cAMP (Kay et al., 1992; Audsley et al., 1993). The leucokinins appear to function independently of cAMP but require cellular Ca^{2+} (Davies, et al., 1995; O'Donnell et al., 1996), suggesting an alternate cellular second messenger pathway. Based on this information we purpose that the mosquito diuresin will increase intracellular cAMP in isolated Malpighian tubules and the mosquito leucokinins will increase tubule IP_3.

In this paper we report the effects of the mosquito diuresin and leucokinins, *A. aegypti* leucokinin peptides (ALP) and the culekinin depolarizing peptides (CDP) on the cellular second messengers cAMP and IP_3 in the Malpighian tubules of *A. aegypti*.

MATERIAL AND METHODS

Animals

Mosquitoes, *A. aegypti* (Rock strain) were raised in small numbers as needed for individual experiments as described (Shapiro and Hagedorn, 1982), or in large numbers in shallow pans containing 3,000 larvae (Wheelock et al., 1991). Mosquitoes were maintained at 27° C on 3% sucrose solution in 16L:8D photoperiod. Animals used in experiments were non-blood fed, adult, female mosquitoes, collected 3-7 days after ecolsion and dehydrated for 12 hours prior to experiments.

Optimization of tissue extraction efficiency for second messengers

Tubules were disrupted in different extraction solvents containing radiolabeled cAMP [5',8-^{3}H] adenosine 3',5'-cyclic phosphate, ammonium salt, with a specific activity of 143 mCi/mg (Amersham Corporation, Arlington Heights, IL). An optimum extraction efficiency of 90.8 ± 1.7% (n = 4 extraction trials) for cAMP was obtained with ethanol:concentrated hydrochloric acid (60:1, v:v).

Tubules were disrupted in various extraction solvents containing D-myo-[^{3}H] inositol 1,4,5-trisphosphate (Amersham International, Arlington Heights, IL), with a specific activity of 42 Ci/mmol. Although high extraction efficiency (96.8 ± 2.2%) was obtained with chloroform/methanol/concentrated HCL (40:80:1, v:v:v), this solvent system resulted in interference in the IP_3 binding protein assay. To avoid interference, tubules were extracted with 15% trichloroacetic acid (TCA) followed by a tri-n-octylamine/freon (1:1, v:v) wash to neutralize the extract. Recovery for the TCA extraction tri-n-octylamine/freon wash was 95.5 ± 3.3% (n = 3 extraction trials).

In Vitro Bioassay for Stimulation of Malpighian Tubule Intracellular cAMP

Mosquitoes were cold anesthetized and Malpighian tubules were removed from the abdomen and isolated from other tissues. Tubules were transferred into 10 μl of a *Aedes* saline solution. Following a 10 minute equilibration period without agonists, tubules were transferred to 10 μl of saline containing agonists with isobutylmethylxanthine (IBMX) at 5 X 10^{-4} M/L (22.2 μg/ml) and incubated for 30 minutes in a humidified chamber at 28°C. IBMX a phosphodiesterase (PDE) inhibitor, was used to enhance intracellular cAMP concentration during incubations. Previous evidence suggests that PDE dependent turnover of cAMP is accelerated in stimulated Malpighian tubules (Coast et al., 1991) and in order to resolve differences in cAMP concentrations a PDE inhibitor was useful. Control tubules were treated as above except that the final incubation was in saline without agonists. Incubation was terminated with the addition of 200 μl of ice cold absolute ethanol (ETOH)/concentrated hydrochloric acid (HCl) (60:1,v:v). Tissue disruption and extraction were completed with bath sonication (Branson, Danbury, CT) for 15 minutes at 4° C. Tubule extracts were centrifuged for 20 minutes at 10,000 g at 4° C. The supernatant was transferred to a new vial and taken to dryness using vacuum centrifugation (Savant Instruments Inc, Farmingdale, NY).

In Vitro Bioassay for Stimulation of Malpighian Tubule Intracellular IP_3

Isolated Malpighian tubules were placed into 10 μl of *Aedes* saline in a small polypropylene centrifuge vial and equilibrated at room temperature for 5 minutes. Agonists, in 5 μl of saline were added to the tubules, vortex mixed and incubated for 5 or 30 seconds at room temperature. Preliminary experiments using ALP-III (4.73 X 10^{-6} M/L, 6.95 μg/ml) determined that the optimal incubation time for IP_3 response in isolated tubules was 5 seconds (Table 3.1). Five second incubations were used for all subsequent in vitro

experiments with peptide agonists. Similar experiments determined that 30 seconds was an optimal incubation time for tubule IP_3 response to 5-hydroxytryptamine (5-HT).

Table 3.1. Effect of *A. aegypti* leucokinin III on tubule IP_3 with time

Time after peptide addition (seconds)	Mean IP_3 (pmoles ± SEM)	n	P
0	3.12 ± 0.62	3	
5	8.14 ± 0.58	3	<0.05
30	5.11± 0.01	3	NS
60	3.27 ± 0.37	3	NS
300	2.34 ± 0.18	3	NS

Means are ± SEM
Values of P were determined by the statistical application of the students t-test for control and experimental group
n equals 60 tubules each (12 mosquitoes)

Incubations were terminated with the addition 200 μl of ice cold 15% trichloroacetic acid (TCA) while vortex mixing. Constant microprobe sonication (Branson, Danbury, CT) for 30 seconds on ice disrupted tubules and extracted inositol 1,4,5-trisphosphate (IP_3). Extracts were centrifuged at 10,000 g for 20 minutes at 4° C. The supernatant was transferred to a new centrifuge vial and 62.5 μl of 10 mM/L ethylenediaminetetraacetic acid (EDTA) and 200 μl of 1,1,2-trichlorotrifluoroethane (freon)/tri-n-octylamine (1:1,v:v) was added and the solution vortex mixed for 30 seconds. Extracts were centrifuged at 10,000 g for one minute at 4° C to improve phase separation. The top phase was pooled and transferred to a new centrifuge vial and taken to dryness by vacuum centrifugation. Dried extracts were stored at -80° C until assayed for IP_3.

Samples for the second messenger in vitro experiments

Samples in second messenger in vitro experiments were obtained from groups of Malpighian tubules. Following stimulation with agonists or saline without agonists for controls, three groups of 20 tubules were combined prior to assay for IP_3. Three groups of 15 tubules were combined prior to assay for cAMP. Following extraction and drying, samples were taken from these larger pools for measurement of IP_3 and cAMP.

Assay for cAMP

cAMP was measured with a competitive enzyme-linked immunosorbent assay (ELISA) according to Kingan (1989) but with modifications. ELISA microtiter plates (Corning Glass Works, Corning, NY) were coated with a cAMP-thyroglobulin conjugate according to Kingan (1989) and diluted to 1:10,000 in 0.01 M/L phosphate buffered saline (PBS, pH 7.4) for 3 hours at 37° C with shaking. Plates were washed with 0.05% polyoxyethylenesorbitanmonolaurate (Tween) in PBS for 5 minutes and blocked overnight at 4° C with 5% nonfat dry milk in PBS.

A serial dilution of cAMP, free acid in 0.05 M/L sodium acetate buffer (pH 6.2) generated a standard curve consisting of points from 0.001 pmoles/well (0.328 pg) to 10 pmoles/well (3.280 X 10^3 pg) (Figure 3.1). A primary antibody dilution of 1:300,000 with an incubation time of 45 minutes at room temperature and a secondary antibody dilution of 1:1000 with incubation of 1 hour resulted in an ED_{50} of 0.04 pmoles.

Dried tubule extracts were reconstituted with 50 μl of sodium acetate buffer. The extracts and standards were acetylated with 3 μl of acetic anhydride:triethylamine (1:2, v:v). Acetylation of samples and standards enhanced assay sensitivity (Kingan, 1989). Aliquots of 15 μl of acetylated extracts and standards were dispensed into cAMP-conjugate coated wells. The primary antibody against cAMP was diluted to 1:300,000 in 85 μl sodium acetate buffer containing 1% normal goat serum (NGS) and added to the wells. The primary antibody against cAMP was provided by Dr. Timothy Kingan. Covered ELISA plates were incubated at room temperature with shaking for 45 minutes, and then washed three times with tween/PBS. The secondary antibody consisting of a peroxidase-conjugated, goat anti-rabbit IgG (Kirkegaard & Perry, Gaithersburg, MD) diluted to 1:1000 in PBS with 1% NGS was added (70 μl) to the wells. Plates were incubated for 60 minutes at room temperature followed by four tween/PBS washes. The plate developer, 3,3',5,5'-tetramethylbenzidine (TMB) and H_2O_2 (1:1, v:v) (Kirkegaard & Perry, Gaithersburg, MD) was added to all wells (75 μl). ELISA plates were covered against light and incubated at room temperature for 30 minutes. The colorometric reaction was stopped with an addition of 75 μl of 1 M/L H_3PO_4 solution. Plates were read at 450 nm with an ELISA plate reader (Molecular Devices Corporation, Menlo Park, CA). A four parameter model of curve fitting was used to calculate the cAMP standard curve with Softmax a data analysis program (Molecular Devices Corporation, Menlo Park, CA).

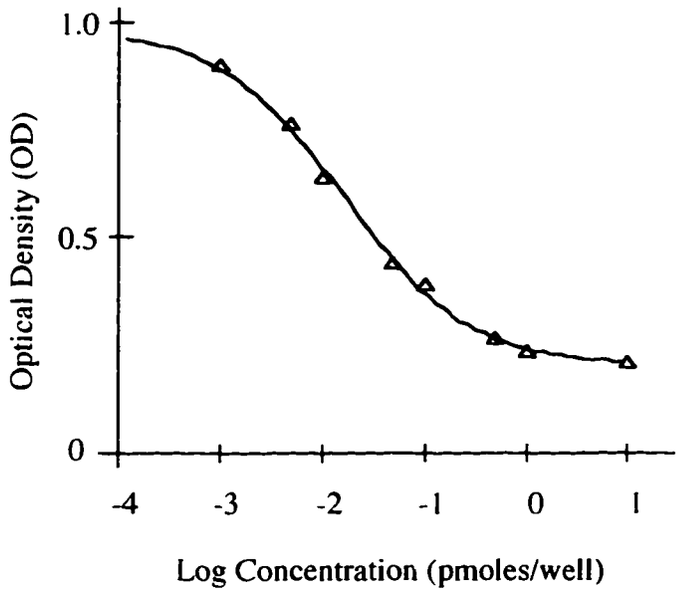

Fig. 3.1. A typical dose response curve for the cAMP ELISA.

Assay for IP3

Isolation of the IP3 binding protein. A sensitive binding protein assay was used for the measurement of tubule IP3 (Palmer and Wakelam, 1990). A standard microsomal preparation was used to isolate a specific IP3 binding protein from bovine adrenocortical tissue. Fresh, whole bovine adrenals (Pel-freez, Rogers, AR) were demedullated and placed into two volumes of chilled 20 mM/L $NaHCO_3$ buffer with 1mM/L dithiothreitol (DTT) at pH 7.5. Demedullated adrenals were chopped in chilled $NaHCO_3$ buffer for one minute in a Waring blender (Waring Products, New Hartford, CT). Chopped adrenal tissue was homogenized on ice for two minutes with an ultra-turrax homogenizer (Tekmar Co., Cincinnati, OH). The homogenate was centrifuged at 5,000 g for 15 minutes at 4° C, the supernatant transferred into a new tube and centrifuged at 35,000 g for 20 minutes at 4° C. The supernatant was discarded and the pellet resuspended in buffer by rapid vortex mixing. The suspension was centrifuged for a second time at 35,000 g for 20 minutes at 4° C. The supernatant was discarded and the pellet resuspended in a small volume of buffer to a final protein concentration of 40 mg/ml. The IP3 binding protein was stored at -80° C. Stock IP3 binding protein prepared from 24 adrenals yielded enough material for 45 assays and remained active for up to 8 months.

The IP_3 binding protein assay. A dilution series of standard IP_3 (Calbiochem-Novabiochem Corporation, La Jolla, CA) was prepared in a solution of 15% TCA washed with freon and tri-n-octylamine (1:1, v:v). This generated a curve sensitive from 0.125 pmol (0.054 ng) to 12.5 pmol (5.473 ng) and was run in parallel with tissue extracts. A final IP_3 binding protein concentration of 5 mg/ml with an incubation time of 45 minutes on ice resulted in an ED_{50} of 1.20 pmoles.

Stock D-myo-[^{3}H] inositol 1,4,5-trisphosphate (Amersham International, Arlington Heights, IL) with a specific activity of 42 Ci/mmol was diluted to 10,000 dpm/25 μl in water. Prior to the assay, stock IP_3 binding protein solution was diluted to 20 mg/ml in 20 mM/L $NaHCO_3$ buffer and stored on ice. Dried tubule extracts were reconstituted in 100 μl of water. Assay components were added to a polypropylene vial (Sarstedt Inc., Newton, NC) in 25 μl volumes; assay buffer (1 M/L Tris-HCl, 60 mM/L NaCl, 0.3 M/L KCl, 3 mM/L EDTA and 3 mg/ml bovine serum albumin (BSA, fraction V), pH 8.3), reconstituted tubule extract, diluted D-myo-[^{3}H] inositol 1,4,5-trisphosphate and IP_3 binding protein solution. Assay vials were vortex mixed and incubated on ice for 60 minutes. Incubation was terminated by centrifugation at 12,000 g for 5 minutes at 4° C. Assay vials were aspirated to remove the supernatant and 200 μl of 1M/L NaOH was added. Vials were incubated for 5 minutes in boiling water to dissolve the pellet containing the bound IP_3. A volume of 1.0 ml of scintillation fluid, Scintiverse Bio HP (Fisher Scientific, Fair Lawn, NJ) was dispensed into each vial followed by rapid vortex mixing. To determine disintegration's per minute vials were counted in a liquid scintillation counter (Beckman Instruments Inc., Fullerton, CA) with external standard and against a prepared quench curve. Standard curve analysis was completed with a logit-log transformation and linear regression using the program Excel (Microsoft Corporation, Redmond, WA) on a Macintosh computer (Apple Computer Inc., Cupertino, CA). A typical standard curve is illustrated in Figure 3.2. All laboratory vials and dispensing apparatus were composed of polypropylene to prevent IP_3 from adhering to lab ware.

Ion Exchange Chromatography

Endogenous cAMP was purified from tubule extracts with anion exchange chromatography. Tubule extracts were passed through mini columns consisting of Dowex AG1 (1X8), 200-400 mesh (BioRad, Rechmond, CA), changed to the formate form and eluted with 2 M/L formic acid. Recovery during preparation of samples averaged 65% as

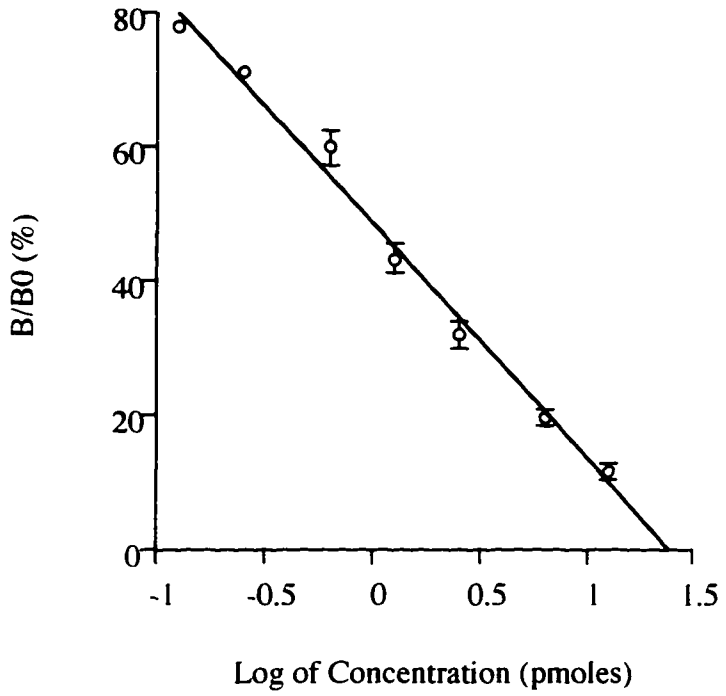

Fig. 3.2. A typical dose curve for the IP_3 binding protein assay.

determined by the addition of radiolabeled cAMP. Endogenous IP_3 was purified from tubule extracts in a similar manner. Extracts were passed through mini columns consisting of Dowex AG1 (1X8), 200-400 mesh (BioRad, Rechmond, CA), changed to the formate form and eluted with 0.8 M/L ammonium formate in 0.1 M/L formic acid. Recovery during preparation of samples for ion chromatography averaged 74.9% based on radiolabeled IP_3.

Protein Assay

Protein concentrations in tubule extracts and IP_3 binding protein preparations were quantified using a BCA protein assay (Pierce Chemical Co., Rockford, IL). Standard curves were prepared with bovine serum albumin (BSA).

Aedes saline

Aedes saline was prepared according to Hagedorn et al., (1977) and consisted of 150 mM/L NaCl, 3.4 mM/L KCl, 1.8 mM/L $NaHCO_3$, 0.6 mM/L $MgCl_2$, 25 mM/L Hepes and adjusted to pH 6.8 - 7.0 with 0.5N NaOH.

Chemicals

D-myo-inositol 1,4,5-trisphosphate and a water soluble forskolin analog, 7b-desacetyl-7b-[γ-(N-methylpiperazino)-butyryl], dihydrocholoride were purchased from Calbiochem-Novabiochem Corporation (La Jolla, CA). Culekinin depolarizing peptides were a gift from Dr. Timothy Hayes, *A. aegypti* leucokinin peptides were provided by Dr. Jan Veenstra (Table 3.2) and *C. salinarius* diuresin was synthesized by Dr. Ron Jasensky (Division of Biotechnology of the Arizona Research Laboratories, University of Arizona, Tucson, AZ) based on the amino acid sequence for the *C. salinarius* diuresin (personal communication Dr. Frank Clottens), Table 3.3. All other chemicals were purchased from Sigma Chemical (St. Louis, MO).

Statistical Analysis

Statistical significance was determined with the Student's t-test using either Excel (Microsoft Corporation, Redmond, WA) or StatView (BrainPower Inc., Calabasas, CA) software.

Table 3.2. The amino acid sequences of the leucokinin related family of peptides

Isolated from, *Leucophaea maderae*											
leucokinin I					D	P	A	**F**	N	**S**	**W** **G-amide**
leucokinin II					D	P	G	**F**	S	**S**	**W** **G-amide**
leucokinin III					D	Q	G	**F**	N	**S**	**W** **G-amide**
leucokinin IV					D	A	S	**F**	H	**S**	**W** **G-amide**
leucokinin V					G	S	G	**F**	S	**S**	**W** **G-amide**
leucokinin VI					E	S	S	**F**	H	**S**	**W** **G-amide**
leucokinin VII					D	P	A	**F**	S	**S**	**W** **G-amide**
leucokinin VIII					G	A	S	**F**	Y	**S**	**W** **G-amide**
Leucokinin-related peptides isolated from, *Culex salinarius*											
Culex leucokinin I							N	P	**F**	H	**S** **W** **G-amide**
Culex leucokinin II				N	N	A	N	V	**F**	Y	**P** **W** **G-amide**
Culex lecuokinin III	W	K	Y	V	S	K	Q	K	**F**	F	**S** **W** **G-amide**
Leucokinin-related peptides isolated from, *Aedes aegypti*											
Aedes leucokinin I	N	S	K	Y	V	S	K	Q	K	**F**	Y **S** **W** **G-amide**
Aedes leucokinin II							N	P	**F**	H	A **W** **G-amide**
Aedes leucokinin III				N	N	P	N	V	**F**	Y	**P** **W** **G-amide**

Bold indicates the amino acids of the shared pentapeptide amide C-terminal region

Table 3.3. The amino acid sequences of the CRF-like diuretic peptides

MasDH	RM**PSLSI**DL**P**MS**VLRQ**K**L**S**L**E**K**E**R**K** VHALR**AA**A**NR**N**FLN**DI-**NH2**
MasDPII	SFSVNPAVDILQHRYMEKV**AQ**N**NR**N**FLN**RV-**NH2**
Locusta	MGMG**PSLSIVNP**M**DVLRQRLLLEIARRR**L**R**DAEE **QI**K**AN**KD**FL**QQ**I-NH2**
Acheta	TGAQ**SLSIVAPLDVLRQRL**MN**E**LN**RRRMRE**LQGSR**IQQNR**QL**L**TS**I-NH2**
Periplnt	TGSG**PSLSIVNPLDVLRQRLLLEIARRRMR**QSQD **QIQANR**E**IL**QT**I-NH2**
Msca/Stx	N**KPSLSIVNPLDVLRQRLLLEIARRQMKE**NTR **Q**VEL**NRAIL**KNV-**NH2**
Culex	T**KPSLSIVNPLDVLRQR**II**LE**M**ARRQMRE**NTR **Q**VER**N**KA**IL**RE**I-NH2**
Tenebrio	TS**P**TI**SI**TA**P**I**DVLR**KTWEQ**E**R**AR**K**QM**VK **NREFLN**SLN-OH
Hum-CRF	SEE**P**PI**S**LDLTFHLLRE V**LE**MARAEQLA **QQA**HS**NR**KLMEI**I-NH2**

Abbreviations: MasDH; *M. sexta* diuretic hormone, MasDPII; *M. sexta* diuretic peptide II, Periplnt; *Periplaneta*, Msca/Stx; *Musca/Stomoxys*, hum-CRF; human CRF
Bold indicates amino acid similarities with three or more sequences

RESULTS

ELISA assay parameters

ELISA non-specific binding was 9.7% calculated by dividing the optical density (OD) of the zero standard without the cAMP conjugate by the OD of the zero standard with the cAMP conjugate. Coefficients of variation for standards were less than or equal to 5 %. Between and within-assay coefficients of variation were 17 and 12 %, respectively (n = 14). Assay coefficient of variation were based on assay controls containing non-radiolabeled cAMP added to pooled tubule tissue extracts.

The cross reactivity for the primary cAMP antibody as determined by Kingan (1989) and was less than 0.01% for the following competing nucleotides: ATP, cGMP, ADP and 5' AMP.

Assay interference

Tubule extracts contained substances which caused interference in the cAMP ELISA. Interfering substances were removed by passing tubule extracts through ion-exchange columns. The mean concentration of cAMP in the initial extracts were 0.11 ± 0.01 pmoles (means ± SE) and after column chromatography, 3.62 ± 0.19 pmoles, n = 4.

Figure 3.3 shows the expected linear increase in cAMP with increasing numbers of tubules and illustrates that contaminating substances were limited to tubule extracts containing

8 or more Malpighian tubules. For all subsequent experiments cAMP was measured in extracts containing 8 or less tubule equivalents and the final concentration was calculated based on allowances for differences in cAMP with increasing tubule equivalents.

The effect of in vitro application of forskolin on isolated Malpighian tubules

Forskolin directly activates adenylate cyclase and was used to determine if the in vitro tubule assay was sensitive enough to resolve stimulated changes in intracellular cAMP. Tubule intracellular cAMP concentrations were significantly increased ($p < 0.01$) in forskolin (10^{-4} M/L) stimulated tubules compared to control 91.4 ± 5.4 pmoles/mg protein vs 6.0 ± 1.8 pmoles/mg protein (mean ± SE), n = 4.

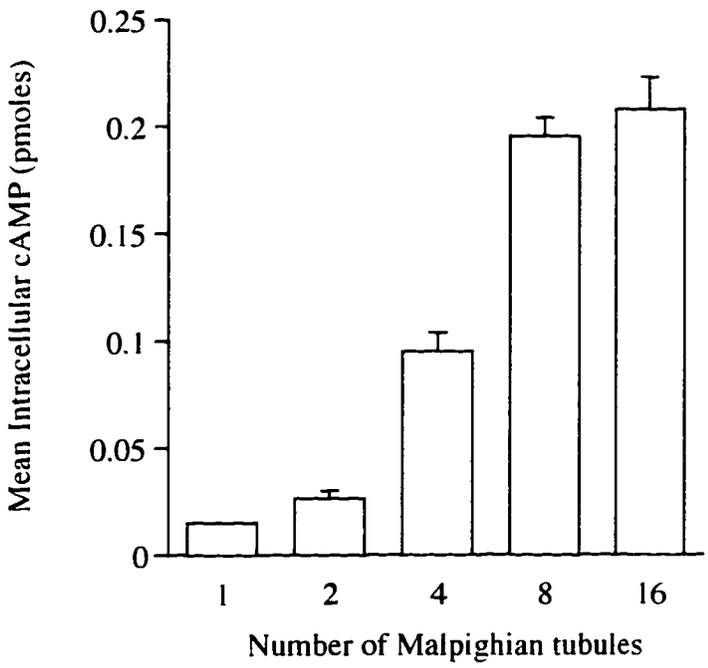

Fig. 3.3. Intracellular cAMP concentrations for increasing Malpighian tubule tissue masses. Each bar represents 4 separate extractions, values represent means ± SE, single Malpighian tubule error bar is too small to appear on the graph.

The effect of in vitro application of 5-hydroxytryptamine on intracellular cAMP in isolated Malpighian tubules

In order to assess tissue viability and monitor changes in sensitivity a positive control was used to evaluate the effect of putative diuretic peptides on intracellular cAMP concentrations in isolated Malpighian tubules. 5-HT (10^{-3} M/L, 3.9 X 10^{-4} g/ml) significantly increased intracellular cAMP in vitro from a baseline concentration of 2.6± 1.3 to 6.7 ± 0.6 pmoles/mg protein, n = 6, $p < 0.05$.

The effect of in vitro application of putative diuretic peptides on intracellular cAMP in isolated Malpighian tubules

The culekinins depolarize isolated Malpighian tubules in adult *A. aegypti* (Hayes et al., 1994), suggesting biological activity. In order to determine the cellular mechanism of

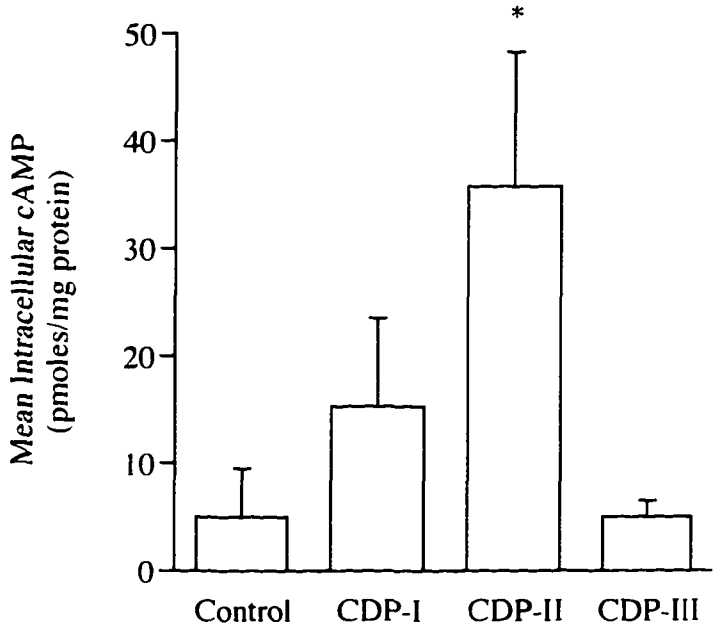

Fig. 3.4. Tubule intracellular cAMP following the in vitro application of culekinin peptide at a final concentration of 2 X 10^{-5} M/L (2.8 X 10^{-5} g/ml) with IBMX. Values represent means ± SE, n = 7, * = $p < 0.05$.

action of these peptides, isolated tubules were treated with culekinins and intracellular cAMP was measured. CDP-1 and CDP-3 did not significantly stimulate intracellular cAMP whereas CDP-2 elevated cAMP levels 7 fold compared to control values (Figure 3.4).

A. aegypti leucokinins have been isolated from adult, headless *A. aegypti* mosquitoes (Veenstra, 1994) and share amino acid sequences with the culekinins (Table 3.2) suggesting similar biological activity. The effects of three *A. aegypti* leucokinins on intracellular cAMP was measured. *A. aegypti* leucokinins did not stimulate tubule cAMP and likely function in a cAMP independent manner (figure 3.5).

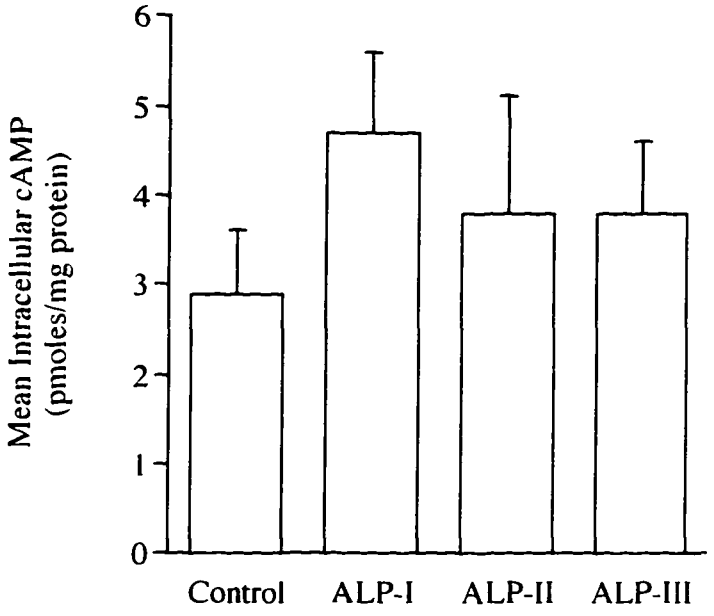

Fig. 3.5 Malpighian tubule intracellular cAMP following the in vitro application of *A. aegypti* leucokinin peptide at a final concentration of (2×10^{-5} M/L, 2.9×10^{-5} g/ml) with IBMX. Values represent means ± SE, n = 9.

In adult *A. aegypti* Malpighian tubules leucokinin VI is the most physiologically active of the leucokinins (Hayes et al., 1989) and leucokinin VI which shares an identical amino acid core sequence with leucokinin IV (Table 3.2) were applied to tubules in vitro. Neither leucokinin IV or VI significantly stimulated intracellular cAMP beyond baseline concentration (Figure 3.6).

These data indicate that some leucokinins (CDP-1, CDP-3, *A. aegypti* leucokinins, leucokinin IV and leucokinin VI) appear to stimulate tubules in a cAMP independent manner, suggesting an alternate cellular signaling mechanism.

In insects, evidence suggests that the diuresins stimulate tubule fluid transport via a cAMP mechanism (Coast et al., 1991, Coast and Kay, 1994, Thompson et al., 1995).

Because of its amino acid sequence homology with the CRF-like diuretic peptides, experiments were designed to determine if mosquito diuresin would stimulated Malpighian tubule solute and fluid transport in a cAMP dependent manner. The *C. salinarius* diuresin was applied at 2 X 10^{-5} M/L (5.2 X 10^{-4} g/ml) to isolated *A. aegypti* Malpighian tubules and significantly increased intracellular cAMP from a baseline concentration of 2.3 ± 0.1 pmoles/mg protein to stimulated levels of 3.2 ± 0.2 pmoles/mg protein, n = 6, ($P < 0.05$). Although these data suggest that this neuropeptide functions via cAMP dependent mechanism, the relatively low response makes the interpretation of these data difficult.

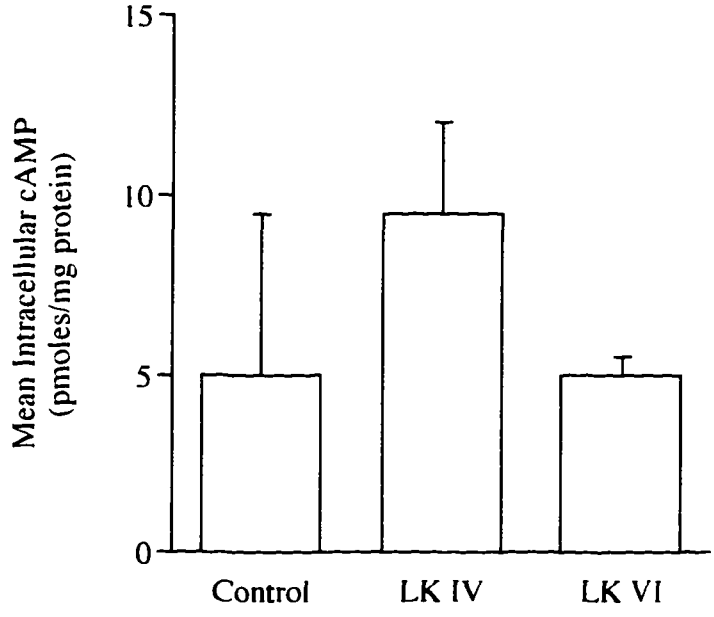

Fig. 3.6. Tubule intracellular cAMP following the in vitro application of leucokinin peptide at a final concentration of 2 X 10^{-5} M/L (2.8 X 10^{-5} g/ml) with IBMX. Values represent means ± SE, n = 6, LK IV (leucokinin IV) and LK VI (leucokinin VI).

IP3 binding protein assay parameters

For the IP3 binding protein assay non-specific binding was 4.7%, calculated as a percentage against total counts. Coefficients of variation for standards were less than or equal to 4.6%. Between and within-assay coefficients of variation were 18 and 10 %, respectively (n=8). Assay coefficients of variation were based on assay controls containing non-radiolabeled IP3 added to tubule extracts.

Cross-reactivity as determined by Palmer and Wakelam (1990) for bovine adrenocortical microsomes as competitive displacement against nanomolar concentrations of radiolabeled inositol 1,4,5-trisphosphate and expressed as EC_{50} concentrations greater than 100 fold above (1,4,5) IP_3 for the following inositol and nucleotide polyphosphates: inositol 2,4,5 trisphosphate, inositol cyclic 1:2,4,5 trisphosphate, inositol 1,3,4 trisphosphate, inositol 1,3,4,5 tetrakisphosphate, inositol 1,3,4,5,6 pentakisphosphate, inositol 1,2,3,4,5,6 hexakisphosphate, adenosine triphosphate (ATP) and guanosine triphosphate (GTP).

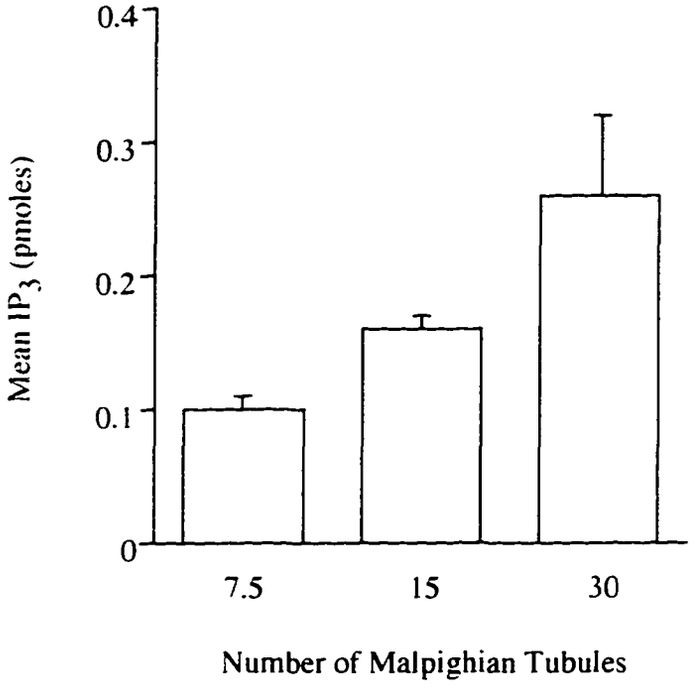

Fig. 3.7 Intracellular IP_3 concentrations for increasing Malpighian tubule tissue masses. Each bar represents the mean ± SE of 3 separate extractions.

Assay interference

There was no significant difference in IP_3 estimates after allowing for recovery losses from pooled Malpighian tubules extracted with 15% TCA (0.22 ± 0.08) and extracts after ion exchange chromatography (0.32 ± 0.05 pmoles) (mean ± SE, n = 3 estimates). There was a linear increase in IP_3 concentration when tubule extracts from increasing numbers of tubules up to 30, were assayed (Figure 3.7). Assay extracts contained no more than 30 tubules per sample.

IP3 binding protein assay compared with a commercial IP3 assay kit

Tubule extracts containing exogenous IP3 were assayed using a modification of the technique of Palmer and Wakelam (1990) and a commercial IP3 assay kit (Amersham International, Arlington Heights, IL). There was no significant difference ($p > 0.05$) in IP3 concentration between the Palmer and Wakelam assay (5.8 ± 0.4 pmoles) and the Amersham IP3 kit (4.2 ± 0.4 pmoles, mean ± SE, n = 3).

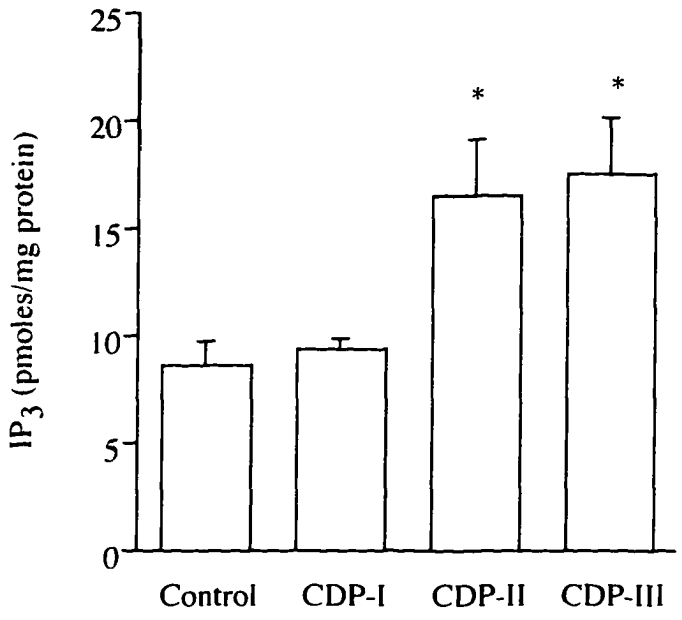

Fig. 3.8 IP3 concentrations for isolated tubules following the addition of culekinin depolarizing peptides at 5 X 10^{-6} M/L. Bars represent mean ± SEM (n = 3), * = $p < 0.05$.

The effect of in vitro application of 5-hydroxytryptamine on intracellular IP3 in isolated Malpighian tubules

Previous evidence suggests that 5-HT may also function in a cAMP independent manner to stimulate Malpighian tubules in insects (Morgan and Mordue, 1984). To determine if 5-HT could stimulate intracellular IP3 in isolated tubules and function as a positive control in the in vitro assay IP3 was measured in tubules following the addition of 5-HT (6.6 X 10^{-4} M/L). 5-HT significantly ($p < 0.05$) increased intracellular IP3 in isolated tubules from a baseline of 2.7 ± 0.3 pmoles/mg protein to a maximum of 6.6 ± 0.4 pmoles/mg protein, mean ± SE (n = 3).

The effect of putative diuretic peptides on intracellular IP3 in isolated Malpighian tubules, in vitro.

Figure 3.8 represents the effects of the culekinin depolarizing peptides on isolated tubules. CDP II and III significantly ($p < 0.05$) stimulated intracellular IP3 above control concentrations. CDP-I did not stimulate tubule IP3 above that of control tubules.

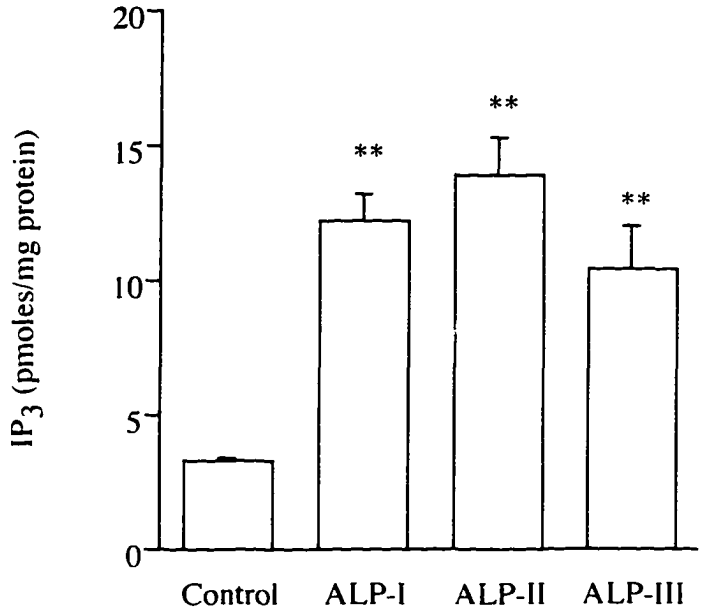

Fig. 3.9 IP3 concentrations for isolated tubules following the addition of *A. aegypti* leucokinins at 5 X 10^{-6} M/L. Bars represent mean ± SEM (n = 3), ** = $p < 0.01$.

Figure 3.9 shows the effect of the in vitro application of the *A. aegypti* leucokinins on tubule IP3 concentrations. All three *A. aegypti* leucokinins significantly ($p < 0.01$) stimulated IP3 in isolated tubules.

The *C. salinarius* diuresin did not stimulate IP3 in isolated tubules. IP3 concentrations following the application of the CFR-like DP (1 X 10^{-6} M/L, 2.5 X 10^{-5} g/ml) were 10.2 ± 1.0 pmoles/mg protein compared to a baseline of 8.5 ± 1.0 pmoles/mg protein, mean ± SE (n = 3), $p > 0.05$.

The effect of lower doses of ALP-I on intracellular IP3 in isolated Malpighian tubules

The application of ALP-I at lower doses to tubules in vitro caused significant stimulation in intracellular IP3 (Table 3.4).

Table 3.4. Effect of ALP-I at lower doses on tubule IP_3.

Dose ALP-I	Mean intracellular IP_3 (pmoles/mg protein ± SEM)	n	P
Control	9.0 ± 0.7	3	
5×10^{-6} M/L	42.8 ± 8.6	3	<0.05
5×10^{-7} M/L	42.3 ± 4.9	3	<0.05
5×10^{-8} M/L	50.3 ± 0.6	3	<0.01

Control represents the addition of saline (without agonist) to the in vitro incubation medium.
Means are ± SEM
Values of p were determined by the statistical application of the students t-test for control and experimental group.

DISCUSSION

The cAMP and IP_3 second messenger assays were demonstrated to be specific, sensitive, precise and accurate. Optimal conditions for assay parameters including non-specific binding, coefficients of variation for assay standards, coefficients of variation for both between and within-assay, assay parallelism, chromatographic purification of endogenous ligand, and determination of extraction recovery losses, were determined for both the cAMP ELISA and the IP_3 binding protein assays. Assay interference in the cAMP ELISA was avoided by reducing the amount of tissue extracted and in the IP_3 binding protein assay by extracting IP_3 from tubules using TCA with a tri-n-octylamine wash.

A positive control agonist for the cAMP and IP_3 experiments was required to evaluate the second messenger response in tubules to putative diuretic peptides. Forskolin was inappropriate as a control agonist for in vitro cAMP experiments because it has been demonstrated to elicit super physiological increases in intracellular cAMP in many tissues via a non receptor mediated process and may affect cAMP independent mechanisms (Laurenza et al., 1989; Baxter and Byrne, 1990). The agonist 5-HT was used because it mediates its biological actions through membrane bound receptors (Maddrell et al., 1971; Maddrell et al., 1991; Saudou et al., 1992; Clark and Bradley, 1997).

The application of 5-HT to isolated *A. aegypti* tubules resulted in a significant stimulation of both intracellular cAMP and IP_3. Previous reports have demonstrated that 5-

HT stimulated fluid secretion in isolated Malpighian tubules in adult *A. aegypti* (Veenstra, 1988), and in larvae (Clark and Bradley, 1996).

It is not surprising that 5-HT stimulates both cAMP and IP_3. In *Calliphora vicina* salivary gland two receptors have been isolated; one activates cAMP and the other functions via a Ca^{2+}/IP_3 pathway (Berridge, 1981; Berridge and Heslop, 1981; Berridge et al., 1983). 5-HT has also been shown to stimulate Malpighian tubules in *Locusta* via a Ca^{2+} dependent pathway (Morgan and Mordue 1984; Morgan and Mordue, 1985).

Endogenous 5-HT has been shown to be significant in regulating diuresis in the blood feeding bug *R. prolixus* (Lange et al., 1989; Maddrell et al., 1991), and in larval *A. aegypti* (Clark, 1994; Clark and Bradley, 1996). Evidence for 5-HT receptors have recently been presented in larval *A. aegypti* tubules (Clark and Bradley, 1997). It is difficult to interrupt the physiological significance of 5-HT in adult *A. aegypti* because of a lack of immunocytochemical evidence for 5-HT neurosecretory cells. The exception to this was the immunocytochemical identification of 5-HT reactive neurons surrounding the salivary gland of the adult female, *A. aegypti* (Novak et al., 1995). Supra physiologic concentrations of 5-HT are required to elicit the secretory response in adult mosquito tubules. The results from this study demonstrate that 5-HT significantly stimulates both second messengers in isolated *A. aegypti* tubules. Taken together with previous work which demonstrated that 5-HT stimulated fluid secretion in tubules (Veenstra, 1988) suggests that this biogenic amine may have a physiological role in modulating tubule function during diuresis.

The *C. salinarius* diuresin stimulated *A. aegypti* tubule cAMP but to lower concentrations than expected. Previous studies have suggested that the diuresins are potent stimulators of tubule fluid secretion and intracellular cAMP (Thompson et al., 1995; Audsley et al., 1995). The mosquito diuresin is an amidated, 44 amino acid peptide containing two methionine residues at positions 22 and 27. Oxidation of methionine residues during storage of some diuresins has been documented (Kataoka et al., 1989; Clottens et al. 1994), and may account for lower than expected tubule cAMP response. Methionine oxidation reduced bioactivity of the *M. sexta* diuretic hormone (Kataoka et al., 1989; Coast et al., 1992) and *Locusta* diuresin (Audsley et al., 1995). In this study the mosquito diuresin was stored for a limited time under similar conditions described by Coast et al. (1992), which led to methionine oxidation and diminished biological activity. *C. salinarius* diuresin analogs containing less reactive groups such as norleucine substituted for methionines could be used to stimulate isolated tubules and determine if loss of biological activity were due to peptide degradation.

The low bioactivity for the *C. salinarius* diuresin may have been due to reduced solubility of the peptide following reconstitution with *Aedes* saline. The synthesized *C. salinarius* CRF-like DP was stored dry with BSA to prevent adsorption and reconstituted with *Aedes* saline prior to use in the in vitro bioassays. Dried *Acheta* diuresin reconstituted in methanol solubilized the peptide more efficiently (Coast and Kay, 1994; Patel et al., 1995). We chose to use *Aedes* saline to prepare peptides for bioassays because dilute methanol might interfere with normal tubule function. If peptide solubility was reduced in saline then lower soluble concentrations of peptide could lead to a reduction in bioactivity.

A. aegypti diuresin has yet to be isolated or synthesized. Amino acid sequence identity between other Dipteran diuresins is greater than 75%. Since *C. salinarius* and *A. aegypti* are closely related, it is reasonable to assume that a high amino acid sequence identity for the mosquito diuresins is possible. In comparing the Dipteran diuresin sequences it is interesting that the N-terminal amino acid is different, asparagine for *M. domestica* and threonine for *C. salinarius* (Table 3). The N-terminal portion of the molecule is important in terms of receptor binding and signal transduction (Audsley et al., 1995), and may be responsible for differences in biological activity. Subtle differences between the *C. salinarius* and the natural *A. aegypti* diuresin could account for lower bioactivity due to differences in receptor-ligand binding preferences. Differences in diuresin receptor ligand affinity have been demonstrated in the insects. The *M. sexta* diuresin receptor has been found to be less ligand restrictive than either the *Acheta* or *Locusta* diuresin receptors (Audsley et al., 1995). If the *A. aegypti* diuresin receptor were ligand restrictive subtle differences in the mosquito diuresins, especially in a critical region of the peptide, could account for lower responses in tubule cAMP with the *C. salinarius* peptide.

The culekinin CDP-I was the only mosquito leucokinin which did not stimulate IP_3 in tubules. CDP-I and ALP-II share nearly identical amino acid sequences, differing only at position five in the C-terminal portion of the peptide. Although IP_3 stimulation by CDP-I was not demonstrated, it is unlikely that it functions via a different cellular mechanism in that CDP-I has been demonstrated to depolarize transepithelial voltage in tubules (Hayes et al., 1994). It is more likely that peptide degradation may have occurred in the in vitro experiments using this small leucokinin. The presence of an alanine in the C-terminal region of ALP-II may render this small leucokinin more resistant to degradation. Alternatively, because the C-terminal pentapeptide region is important in receptor recognition (Hayes et al., 1989), the subtle differences in CDP-I could possibly prevent recognition and binding to the *A. aegypti* receptor.

The *C. salinarius* leucokinin CDP-II was the only mosquito leucokinin that stimulated both tubule intracellular cAMP and IP_3. ALP-III bears close amino acid sequence homology to CDP-II but stimulated only tubule IP_3. ALP-III differs from CDP-II at a single amino acid at position 3 in the N-terminal region of the peptide. CDP-II has an alanine at position 3 as opposed to ALP-III which has a proline. The pentapeptide C-terminal region is identical in both peptides and is important in receptor recognition and biological potency (Hayes et al., 1989). The fact that both peptides stimulated tubule IP_3 but only CDP-II stimulated cAMP, suggests that CDP-II may activate two different receptors because of the subtle differences in the amino acid sequence. It is possible that CDP-II may activate the *A. aegypti* ALP-III receptor and also bind to and activate a less ligand-specific receptor coupled to the adenylate cyclase pathway thereby stimulating both IP_3 and cAMP. The explanation discussed above implies that two different membrane receptors may account for the activation of different cellular messenger systems. It is possible that the actions of CDP-II could involve a single membrane receptor which could possibly activate different cellular signal pathways via an identical G-protein as has been described for a muscarinic receptor system (Baumgold, 1992).

The *A. aegypti* leucokinins and the *C. salinarius* leucokinins, CDP-II and CDP-III stimulated intracellular IP_3 concentration in isolated Malpighian tubules. Fogg et al. (1990), have demonstrated an agonist mediated increase in phospholipase C activity in insect Malpighian tubules. Measurement of increases in tubule IP_3 following treatment with leucokinin agrees with previous studies which suggest that the leucokinins function via a cAMP independent mechanism (Takemura et al., 1989; Coast et al., 1993; Davies et al., 1995; O'Donnell et al., 1996). Although there is no direct evidence for leucokinins altering intracellular Ca^{2+} levels, recent studies using thapsigargin and BAPTA-AM which manipulate intracellular Ca^{2+} suggest that Ca^{2+} is important in mediating the actions of the leucokinins (Davies et al., 1995; O'Donnell et al., 1996). These results suggest that leucokinin stimulation of tubules may lead to transient increases in cellular Ca^{2+} through the release of IP_3-sensitive intracellular Ca^{2+} stores. The role of extracellular Ca^{2+} cannot be ruled out as metabolites from the polyphosphoinositide pathway can involve extracellular Ca^{2+} (Bolander, 1989). Clearly it will be important to define the role of Ca^{2+} in the actions of leucokinins at the tubules. Measurement of cellular Ca^{2+}, possibly using fluorescent Ca^{2+} chelating agents such as Fura-2 combined with appropriate imaging methods would be helpful in determining how the leucokinins influence changes in tubule cells. This method may obviate interference by principal cells of *A. aegypti* tubules which contain abundant

concentric lamellar Ca^{2+} crystals that could interfere with conventional Ca^{2+} imaging techniques (Bradley et al., 1982).

The endocrine regulation and physiology of the Malpighian tubule has recently become better understood. Evidence for an apical V-ATPase proton pump which establishes a proton gradient and operates as a complex with the cation/proton antiporter(s) in the tubules is convincing (Maddrell and O'Donnell, 1992; Klein, 1992; Pietrantonio and Gill, 1992; Wieczorek, 1992; O'Donnell and Maddrell, 1996). Studies suggest that the apical membrane V-ATPase proton pump is probably directly stimulated by cAMP dependent endocrine factor(s) (Maddrell and O'Donnell, 1992; O'Donnell and Maddrell, 1996), possibly via the diuresins. Activation of the apical V-ATPase that is complexed with the cation/proton antiporter(s) leads to an increase in cation movement across the tubule via active transport processes. In order to sustain this process, appropriate counter-anion movement is necessary. The leucokinins which increase passive Cl^- permeability across the tubules (O'Donnell and Maddrell, 1996), may enable rapid anion response and may have an important role in regulating counter-anion flow during transepithial fluid movement. Cation and anion movement across the epithelium are regulated by distinct processes (O'Donnell et al., 1996), and require different endocrine factors, probably operating through diverse cellular signal transduction pathways. Both aspects of this process must function in a coordinated manner to enable fluid movement across the tubules, leading to the formation of the primary urine.

This study provides evidence in support of Ca^{2+} dependent leucokinin stimulation of *A. aegypti* Malpighian tubules. The data demonstrate that leucokinin application to isolated tubules lead to increases in tubule IP_3, probably via receptor mediated activation of the polyphosphoinositide pathway. Increases in cellular IP_3 have been associated with transient increases in cellular Ca^{2+} through the release of intracellular IP_3-sensitive Ca^{2+} stores (Berridge, 1988; Sekar and Hokin, 1986). As endocrine factors, the leucokinins have been shown to increase transepithelial Cl^- conductance across mosquito Malpighian tubules which may function as a counter anion to cation flow leading to fluid movement and production of the primary urine.

Acknowledgments--We thank Dr. Jan Veenstra for providing the ALPs and for his comments and suggestions, Dr. Timothy Hayes for providing the CDPs and Dr. Tim Kingan for providing the cAMP antibody. This work was supported in part by NSF grant number

DCB-8918437, MacArthur Foundation Vector Biology Grant to the Center for Insect Science and the Agricultural Experiment Station at the University of Arizona.

REFERENCES

Audsley N., Coast G. M. and Schooley D. A. (1993) The effects of *Manduca sexta* diuretic hormone on fluid transport by the Malpighian tubules and cryptonephric complex of *Manduca sexta*. *J. Exp. Biol.* **178**, 231-243.

Baumgold J. (1992) Muscarinic receptor-mediated stimulation of adenylyl cyclase. *Trends Physiol. Scie.* **13**, 339-340.

Baxter D. A. and Byrne J. H. (1990) Reduction of voltage-activated K^+ currents by forskolin is not mediated via cAMP in pleural sensory neurons of *Aplysia*. *J. Neurophysiol.* **64** (5), 1474-1483.

Berridge M. J. (1981) Electrophysiological evidence for the existence of separate receptor mechanisms mediating the action of 5-hydroxytryptamine. *Mol. Cell Endocrinol.* **23**, 91-104.

Berridge, M. J. (1988) Inositol lipids and calcium signalling. *Pro. R. Soc. Lond.* **234**, 359-378.

Berridge M. J. and Heslop J. P. (1981) Separate 5-hydroxytryptamine receptors on the salivary gland of the blowfly are linked to the generation of either cyclic adenosine 3',5'-monophosphate or calcium signals. *Br. J. Pharmac.* **73**, 729-738.

Berridge, M. J., Dawson R. M. C., Downes P.C., Heslop J. P. and Irvine R. F. (1983) Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. *Biochem. J.* **212**, 473-482.

Blackburn M. (1991) Isolation and identification of a new diuretic peptide from the tobacco hornworm, *Manduca sexta*. *Biochem. Biophys. Res. Commun.* **181**, 927-932.

Blackburn M. B., Wagner R. M., Shabanowitz J., Kochansky J. P., Hunt D. F. and Raina A. K. (1995) The isolation and identification of three diuretic kinins from the abdominal ventral nerve cord of adult Helicoverpa zea. *J. Insect Physiol.* **41**, 723-730.

Bolander F. F. (1989) Calcium, calmodulin and phospholipids. In: *Molecular Endocrinology. 1989*, pp 150-152 Academic Press, San Diego.

Cantera R., Hansson B. S., Hallberg E. and Nassel D. R. (1992) Postembryonic development of leucokinin I-immunoreactive neurons innervating a neurohemal organ in the turnip moth *Agrotis segetum*. *Cell Tissue Res.* **269**, 65-77.

Chen Y., Veenstra J. A., Davis N. T. and Hagedorn H. H. (1994a) A comparative study of leucokinin-immunoreactive neurons in insects. *Cell Tissue Res.* **276**, 69-83.

Chen Y., Veenstra J. A., Hagedorn H. and Davis N. T. (1994b) Leucokinin and diuretic hormone immunoreactivity of neurons in the tobacco hornworm, *Manduca sexta*, and co-localization of this immunoreactivity in lateral neurosecretory cells of abdominal ganglia. *Cell Tissue Res.* **278**, 493-507.

Clark T. M. (1994) Ph.D. Dissertation, University of California, Irvine. Hormonal control of Malpighian tubules in larval *Aedes aegypti*.

Clark T. M. and Bradley T. J. (1996) Stimulation of Malpighian tubules from larval *Aedes aegypti* by secretagogues. *J. Insect Physiol.* **42**, 593-602.

Clark T. M. and Bradley T. J. (1997) Malpighian tubules of larval *Aedes aegypti* are hormonally stimulated by 5-HT in response to increased salinity. *Arch. Insect Biochem. and Physiol.* **34**, 123-141.

Coast G. M. and Kay I. (1994) Effect of *Acheta* diuretic peptide on isolated Malpighian tubules from the house cricket *Acheta domesticus*. *J. Exp. Biol.* **187**, 225-243.

Coast G. M., Holman G. M. and Nachman R. J. (1990) The diuretic activity of a series of cephalomyotropic neuropeptides, the achetakinins, on isolated Malpighian tubules of the house cricket, *Acheta domesticus*. *J. Insect Physiol.* **36**, 481-488.

Coast G. M., Cusinato O. Kay I. and Goldsworthy G. J. (1991) An evaluation of the role of cyclic AMP as an intracellular second messenger in Malpighian tubules of the house cricket, *Acheta domesticus*. *J. Insect Physiol.* **37**, 563-573.

Clottens F. L., Holman M. G., Coast G. M., Totty N. F., Hayes T. K., Kay I., Mallet A. I., Wright M. S., Chung J. S., Truong O. and Bull D. L. (1994) Isolation and characterization of a diuretic peptide common to the house fly and stable fly. *Peptides*. **15**, 971-979.

Davies S. A., Huesmann G. R., Maddrell S. H. P., O'Donnell M. J., Skaer N. J. V., Dow J. A. T. and Tublitz N. J. (1995) CAP2b, a cardioacceleratory peptide, is present in Drosophila and stimulates tubule fluid secretion via cGMP. *Am. J. Physiol.* **269**, R1321-R1326.

Digan M. E., Roberts D. N., Enderlin F. E., Woodworth A. R. and Kramer S. J. (1992) Characterization of the precursor for *Manduca sexta* diuretic hormone Mas-DH. *Proc. Natl. Acad. Sci.* **89**, 11074-11078.

Fogg K., Anstee J. and Hyde D. (1990) Effects of corpora cardiaca extract on intracellular second messenger levels in Malpighian tubules of *Locusta migratoria* L. *J. Insect Physiol.* **36**, 383-389.

Furuya K., Schegg K.M., Wang H., King D.D., and Schooley D.A. (1995) Isolation and identification of a diuretic hormone from the mealworm *Tenebrio molitor*. *Proc. Natl. Acad. Sci. USA* **92**, 12323-12327.

Hagedorn, H. H, Turner, S., Hagedorn, E. A., Pontecorvo, D., Greenbaum, P., Wheelock, G. and Flanagan, T.R. (1977) Postemregence growth of the ovarian follicles of Aedes aegypti. *J. Insect. Physiol.* **23**, 203-

Hayes T. K., Pannabecker T. Hinckley D. Holman G. Nachman R. Petzel D. and Beyenbach K. W. (1989) Leucokinins, a new family of ion transport stimulators and inhibitors in insect Malpighian tubules. *Life Sci.* **44**, 1259-1266.

Hayes T. K., Holman G. M. Pannabecker T. L. Wright M. S. Strey A. A. Nachman R. J. Hoel D. F. Olson J. K. and Beyenbach K.W. (1994) Culekinin depolarizing peptide: a mosquito leucokinin-like peptide that influences insect Malpighian tubule ion transport. *Regul. Pept.* **52**, 235-248.

Holman G. M., Cook B. J. and Nachman R. J. (1986) Primary structure and synthesis of a blocked myotropic neuropeptide isolated from the cockroach, *Leucophaea maderae*. *Comp. Biochem. Physiol.* **85**, 219-224.

Holman G. M., Nachman R. J. and Wright M. S. (1990) Comparative aspects of insect myotropic peptides. *Prog. Clin. Biol. Res.* **342**, 35-39.

Kataoka H., Troetschler R., Li J., Kramer S., Carney R. and Schooley D. (1989) Isolation and identification of a diuretic hormone from the tobacco hornworm, *Manduca sexta*. *Proc. Natl. Acad. Sci.* **86**, 2976-2980.

Kay I., Coast G. M. Cusinato O. Wheeler C. H. Totty N. F. and Goldsworthy G. J. (1991a) Isolation and characterization of a diuretic peptide from *Acheta domesticus*, evidence for a family of insect diuretic peptides. *Biol. Chem. Hoppe-Seyler.* **372**, 505-512.

Kay I., Patel M., Coast G. M., Totty N. F., Mallet A. I. and Goldsworthy G. J. (1992) Isolation, characterization and biological activity of a CRF-related diuretic peptide from *Periplaneta americana* L. *Regul. Pept.* **42**, 111-122.

Kay I., Wheeler C. H. Coast G. M. Totty N. F. Cusinato O. Patel M. and Goldsworthy G. J. (1991b) Characterization of a diuretic peptide from *Locusta migratoria*. *Biol. Chem. Hoppe-Seyler*. **372**, 929-934.

Kingan T. G. (1989) A competitive enzyme-linked immunosorbent assay: Applications in the assay of peptides, steroids, and cyclic nucleotides. *Anal. Biochem.* **183**, 283-289.

Laurenza A., Sutkowski E. M. and Seamon K. B (1989) Forkolin: a specific stimulator of adenylyl cyclase or a diterpene with multiple sites of action? *Trends Pharmacol. Sci.* **10**, 442-447.

Lehmberg E., Ota R., Furuya K., King D., Applebaum S., Ferenz H. and Schooley D. (1991) Indentification of a diuretic hormone of *Locusta migratoria*. *Biochem. Biophys. Res. Commun.* **179**, 1036-1041.

Maddrell S. H. P., Herman W. S. Mooney R. L. and Overton J. A. (1991) 5-Hydroxytryptamine: A second diuretic hormone in *Rhodnius prolixus*. *J. exp. Biol.* **156**, 557-566.

Morgan P. J. and Mordue W. (1984) 5-Hydroxytryptamine stimulates fluid secretion in locust Malpighian tubules independently of cAMP. *Comp. Biochem. Physiol.* **79C**, 305-310.

Morgan P. and Mordue W. (1985) Cyclic AMP and locust diuretic hormone action. *Insect Biochem.* **15**, 247-257.

Nassel D. R., Cantera R. and Karlsson A. (1992) Neurons in the cockroach nervous system reacting with antisera to the neuropeptide leucokinin I. *J Comp Neurol* **322**, 45-67.

Novak M. G., Ribeiro J. M. and Hildebrand J. G. (1995) 5-hydroxytryptamine in the salivary glands of adult female *Aedes aegypti* and its role in regulation of salivation. *J. Exp. Biol.* **198**, 167-174.

O'Donnell M.J., Dow J.A.T., Huesmann G.R., Tublitz N.J. and Maddrell S.H.P. (1996) Separate control of anion and cation transport in Malpighian tubules of Drosophila melanogaster. *J. exp. Biol.* **199**, 1163-1165.

Palmer S. and Wakelam M. J. O. (1990) Mass measurement of inositol 1,4,5-trisphosphate using a specific binding protein assay. In: Methods in insositide research, (Ed. Irvine R. F.), pp 127-134. Raven Press, New York.

Pannabecker T. L., Hayes T. K. and Beyenbach K. W. (1993) Regulation of epithelial shunt conductance by the peptide leucokinin. *J. Membr. Biol.* **132**, 63-76.

Patel M., Chung J. S., Kay I., Mallet A. I., Gibbon C. R., Thompson K. S. J., Bacon J. P. and Coast G. M. (1994) Localization of Locusta-DP in locust CNS and hemolymph satifsies initial hormonal criteria. *Peptides* **15**, 591-602.

Phillips J. (1981) Comparative physiology of insect renal function. *Am. J. Physiol.* **241**, R241-R257.

Reagan J. D. (1994) Expression cloning of an insect diuretic hormone receptor. *J. Biol. Chem.* **261**, 9-12.

Schoofs L., Holman G. M., Proost P., Damme J. V., Hayes T. K. and Loof A. D. (1992) Locustakinin, a novel myotropic peptide from *Locusta migratoria*, isolation, primary structure and synthesis. *Regul. Pept.* **37**, 49-57.

Sekar M. C. and Hokin L. E. (1986) The role of phosphoinositides in signal transduction. J. Memb. Biol. **89**, 193-210.

Shapiro, J. P. and Hagedorn, H. H. (1982) Juvenile hormone and the development of ovarian responsiveness to a brain hormone in the mosquito, *Aedes aegypti. Gen. Comp. Endocrinol.* **46**, 176-183.

Takemura H., Hughes A. R. Thastrup O. and Putney J. W. (1989) Activation of calcium entry by the tumor promoter thapsigargin in parotid acinar cells. *J. Biol. Chem.* **264**, 12266-12271.

Thompson, K. S. J., Rayne, R. C., Gibbon, C. R., May, S. T., Patel, M., Coast, G. M. and Bacon, J. P. (1995) Cellular colocalization of diuretic peptides in locusts: a potent control mechanism. *Peptides*. **16**, 95-104.

Veenstra J. A. (1988) Effects of 5-hydroxytryptamine on the Malpighian tubules of *Aedes aegypti. J. Insect Physiol.* **34**, 299-304.

Veenstra J. A. (1994) Isolation and identification of three leucokinins from the mosquito *Aedes aegypti. Biochem. Biophys. Res. Comm.* **202**, 715-719.

Veenstra J. A. and Hagedorn H. H. (1991) Indentification of neuroendocrine cells producing a diuretic hormone in the tobacco hornworm moth, *Manduca sexta. Cell Tissue Res.* **266**, 359-364.

Wheelock, G. H., Sieber, K. P. and Hagedorn, H. H. (1991) Rapid isolation of a neurohormone from mosquito heads by high-performance liquid chromatography. *J. Chromatog.* 542, 508-514.

Wheelock G., Petzel D., Gillett J. D., Beyenbach K. W. and Hagedorn H. H. (1988) Evidence for hormonal control of diuresis after a blood meal in the mosquito *Aedes aegypti. Arch. Insect Biochem. Physiol.* **7**, 75-89.

Williams J. and Beyenbach K. W. (1983) Differential effects of secretagogues on Na and K secretion in the Malpighian tubules of *Aedes aegypti* (L.). *J. Comp. Physiol.* **149**, 511-517.

4. SUMMARY AND CONCLUSIONS

5-Hydroxytryptamine

In *R. prolixus*, 5-HT acts synergistically with an uncharacterized diuretic factor to regulate Malpighian tubule function after a blood meal (Lange et al., 1989; Maddrell et al., 1991). In larval *A. aegypti*, 5-HT acts on the tubules and functions to regulate ion and water balance (Clark and Bradley, 1994; Clark and Bradley, 1996). The role of 5-HT in tubule function has not been defined in the adult mosquito. Other investigators have demonstrated similar responses to 5-HT in isolated tubules of adult (Veenstra, 1988) and larval *A. aegypti* (Clark and Bradley, 1996). It has been demonstrated here that 5-HT significantly increased the rate of urine production in a dose dependent manner in adult female *A. aegypti* in vivo. The ED_{50} values for the 5-HT injection experiments were 100 fold higher than all other agonists tested, suggesting a relatively low affinity between 5-HT and its receptor(s). Doses greater than 2×10^{-4} μmoles inhibited urine production, suggesting either receptor down regulation, or that more than one type of 5-HT receptor is active. 5-HT receptors have not been characterized in many insects. Multiple 5-HT receptor subtypes have been recognized in *D. melanogaster* (Saudou et al., 1992). *A. aegypti* tubules may possess multiple subtypes of the 5-HT receptor. Alternatively, 5-HT injections above 2×10^{-4} μmoles could influence either the midgut or the rectum. Higher doses of 5-HT could enhance solute and water movement across the midgut resulting a dilution of 5-HT titers in the hemolymph, preventing 5-HT stimulation of the Malpighian tubules. Increased doses of 5-HT could stimulate the rectal absorption resulting in an increase in solute and fluid transport from the rectal lumen into the hemolymph. Stimulation of either the midgut or the rectum with higher doses of 5-HT would likely result in lower total urine production, providing a possible explanation for reduced urine volumes at higher 5-HT doses.

In the present study 5-HT significantly stimulated two intracellular second messengers, cAMP and IP_3. Activation of two different intracellular messengers suggests the possibility of at least two 5-HT receptors in the mosquito tubule. Two 5-HT receptors have been isolated in *C. vicina* salivary gland; one activating intracellular cAMP and the other functioning via a Ca^{+2}/IP_3 pathway (Berridge and Heslop, 1981; Berridge, 1981; Berridge et al., 1983). 5-HT stimulated Malpighian tubules in *L. migratoria* via a Ca^{2+} dependent pathway (Morgan and Mordue 1984, 1985). Veenstra (1988), showed distinct

response differences between 5-HT and cAMP and two of the diuretic factors isolated from *A. aegypti* head extracts, factor II and III. Factor II, III and cAMP stimulated a Na^+ rich tubule fluid while 5-HT simulated a fluid with a slightly increased K^+ concentration (Williams and Beyenbach, 1983; Petzel, 1985; Veenstra, 1988). 5-HT probably activates two distinct receptors in the tubules, one acting through an adenylate cyclase coupled receptor and the other functioning through a Ca^{2+}/IP_3 cellular signal transduction pathway.

Extremely high doses of 5-HT were required to elicit detectable increases in urine production and second messenger concentrations, complicating the assessment of the role of 5-HT in tubule function in the present study. Stimulation of urine production in a dose dependent manner, and the activation of intracellular second messengers, indicates biological activity. 5-HT may modulate tubule function, however, it is questionable whether 5-HT has a significant role in the regulation of urine production in the adult mosquito. In *A. aegypti*, only neurons surrounding the salivary glands have been identified immunocytochemically to contain 5-HT (Novak et al., 1995). Until 5-HT receptors and relevant neurohemal sites are identified, the physiological role of 5-HT in the adult mosquito tubule will remain poorly defined.

The mosquito diuresin

The mosquito diuresin significantly stimulates increased urine production in a dose dependent manner in vivo. The mosquito diuresin also increased intracellular cAMP in isolated tubules of *A. aegypti*. Urine production increases were less than that of the leucokinins. In previous studies, diuresins in other insects significantly stimulated tubule fluid secretion and intracellular cAMP (Coast et al., 1992; Thompson et al., 1995; Audsley et al., 1995).

The amino acid sequence identity between the Dipteran diuresins, *C. salinarius* versus *M. domestica* is greater than 75%. Although the *A. aegypti* diuresin has yet to be isolated and sequenced, it will likely exhibit close amino acid sequence homology with the peptide from *C. salinarius*. The N-terminal amino acid differs between the *M. domestica* and *C. salinarius* diuresin sequences, asparagine for *M. domestica* and threonine for *C. salinarius*. The N-terminal region of this peptide is important in determining receptor-ligand binding affinity and signal transduction response (Audsley et al., 1995). Differences in amino acid residues between the *C. salinarius* diuresin and the native *A. aegypti* peptide at the N-terminal region could alter ligand-receptor affinity and result in lower biological activity. Differences in receptor binding affinity for some insect diuresins

has been demonstrated. Audsley et al. (1995) have reported that the *M. sexta* diuresin receptor is less ligand specific, compared to either the *A. domesticus* or *L. migratoria* diuresin receptors.

Technical problems may have contributed to the reduced biological activity of the diuresin in the bioassays. The *C. salinarius* diuresin peptide contains two methionine residues at positions 22 and 27. Methionine oxidation has been documented for the *M. domestica* diuresin (Clottens et al., 1994) and has been shown to reduce biological activity of the *M. sexta* diuresin (Kataoka et al., 1989; Coast et al., 1992) and the *L. migratoria* diuresin (Audsley et al., 1995). In the present study the mosquito diuresin was stored for a limited time under conditions described by Coast et al. (1992), that led to methionine oxidation and diminished biological activity. *C. salinarius* diuresin analogs, which have fewer reactive groups (such as those that have norleucine in place of methionine), could be used in our bioassays to assess whether the loss of biological activity was due to peptide degradation.

The low bioactivity of the *C. salinarius* diuresin may have been due to reduced solubility of the peptide following reconstitution with *Aedes* saline. In our experiments, the synthesized *C. salinarius* diuresin was stored dry with BSA to prevent adsorption and then reconstituted with *Aedes* saline for use in the bioassays. Dried *A. domesticus* diuresin, reconstituting in methanol, solubilized the peptide more efficiently (Coast and Kay, 1994; Patel et al., 1995). We chose to use *Aedes* saline to prepare peptides for bioassays because dilute methanol might interfere with normal tubule function. If peptide solubility was reduced, lower soluble concentrations of peptide could lead to a reduction in bioactivity.

Mosquito leucokinins

The mosquito leucokinins increased urine production in a dose dependent manner in vivo. Mosquito leucokinins were ranked according to the estimated ED_{50} values of the dose response curve, from high to low, CDP-I > CDP-III > ALP-II > CDP-II > ALP-I > ALP-III. Although differences between ED_{50} values for the leucokinins were not statistically significant, ranking peptides in this manner suggests a trend in agonist/receptor binding affinity (Coast et al., 1990). The high and low ends of this range may demonstrate broad differences in binding affinity. CDP-I was at the high end of the ED_{50} range, indicating relatively low receptor binding affinity compared to the other mosquito leucokinins. CDP-I and ALP-II share amino acid sequence similarities, but vary at position

five of the C-terminal pentapeptide core sequence. The C-terminal pentapeptide region is important for receptor recognition Hayes et al. (1989), and differences in CDP-I at a single amino acid residue could possibly reduce recognition and binding to the *A. aegypti* receptor. ALP-II represents the endogenous *A. aegypti* peptide; it is reasonable that it should have a high receptor binding affinity relative to the *C. salinarius* leucokinin. In general, the *A. aegypti* leucokinin peptides were at the lower end of the ED_{50} ranking range, suggesting that the endogenous peptides tended to have higher receptor binding affinities, compared to the culekinins. This is most apparent with ALP-III which had a 10 fold lower ED_{50} compared to the other mosquito leucokinins tested.

It is interesting to note that CDP-I was the only mosquito leucokinin which did not stimulate intracellular second messengers in isolated tubules. This suggests that CDP-I used in the second messenger experiments may have degraded or conditions within the experiments were not conducive to cross reactive binding for the *C. salinarius* leucokinin. Although, CDP-I and ALP-II both significantly increased urine production this effect may have occurred at different sites within the alimentary system. ALP-I stimulated tubule second messenger, IP3, suggesting that it's influence on urine production was probably on the tubules. CDP-I could increase urine production by acting on the rectum, functioning by inhibiting water reabsorption and ultimately leading to subtle increases in urine production. The role of the rectum in influencing the final urine is reduced at higher urine flow rates Williams and Beyenbach (1983), but at lower flow rates, like those induced by leucokinins, the role of the rectum may be more important.

Immunization of mosquitoes with polyclonal antisera against either ALP-I or *C. salinarius* diuresin resulted in significant inhibition of peptide-stimulated urine production. These data suggest that the antisera remained active and capable of binding and inactivating the appropriate peptide. Previous studies have demonstrated that injected antiserum remains functional in insects for at least 12 hours (Patel et al., 1995). In these studies, the action of the *L. migratoria* diuresin on the locust tubule was blocked by injection of an anti-diuresin antiserum. The peptidergic action of cardioactive neuropeptide was inhibited with a monoclonal antibody directed against this peptide in *M. sexta* (Tublitz, 1988). As reported here, inhibition caused by injected antiserum was not to baseline urine production levels, which may have been due to sub-optimal antibody titers or partial degradation of the antiserum during the extended incubation period. The inhibition of urine production following antibody treatment suggests that ALP-I and *C. salinarius* diuresin stimulates urine production in the mosquito.

The *C. salinarius* leucokinin CDP-II was the only mosquito leucokinin that stimulated both tubule intracellular cAMP and IP3. ALP-III bears close amino acid sequence homology to CDP-II but stimulated only tubule IP3. ALP-III differs from CDP-II at a single amino acid at position 3 in the N-terminal region of the peptide; CDP-II has an alanine at position 3 as opposed to ALP-III which has a proline. The pentapeptide C-terminal region is identical in both peptides and is important in receptor recognition and biological potency (Hayes et al., 1989). The fact that both peptides stimulated tubule IP3, but only CDP-II stimulated cAMP suggests that CDP-II may activate two different receptors. It is possible that CDP-II may activate the *A. aegypti* ALP-III receptor and also bind to and activate a less specific receptor that is coupled to the adenylate cyclase pathway thereby stimulating both IP3 and cAMP. CDP-II had relatively high receptor binding affinity, compared to the other *C. salinarius* leucokinins. This is not surprising as CDP-II was unique in its ability to stimulate both cAMP and IP3 in isolated Malpighian tubules of *A. aegypti*. Stimulation of two second messenger pathways suggests that two different receptors may be activated, which could possibly account for the higher receptor affinity. Stimulation of two cellular signal mechanisms may result from the activation of multiple transport mechanisms in the Malpighian tubule (O'Donnell et al., 1996) and may explain why CDP-II stimulated a higher urine production rate. It is possible that the actions of CDP-II could involve a single membrane receptor which could activate different cellular signal pathways via an identical G-protein as has been described for a muscarinic receptor system (Baumgold et al., 1992).

The *A. aegypti* leucokinins and the *C. salinarius* leucokinins, CDP-II and CDP-III increased intracellular IP3 concentration in isolated Malpighian tubules. Fogg et al. (1990), have demonstrated an agonist-mediated increase in phospholipase C activity in insect Malpighian tubules, using brain extracts. Measurement of increases in tubule IP3 following treatment with leucokinin agrees with previous studies which suggest that the leucokinins function via a cAMP independent mechanism (Coast et al., 1993; Davies et al., 1995; O'Donnell et al., 1996). Although there is no direct evidence for leucokinins altering intracellular Ca^{2+} levels, recent studies using thapsigargin and BAPTA-AM which manipulate intracellular Ca^{2+} suggest that Ca^{2+} is important in mediating the actions of the leucokinins (Davies et al., 1995; O'Donnell et al., 1996). Considered together with data presented in this study, the leucokinins appear to function at the Malpighian tubules of *A. aegypti* by increasing intracellular Ca^{2+} through the release of IP3 sensitive intracellular Ca^{2+} stores. In further investigations concerning the role of the leucokinins in the

mosquito, it will be necessary to directly measure changes in intracellular Ca^{2+} following peptide application. With the availability of fluorescent Ca^{2+} chelating agents such as fura-2, these data may soon be available. Membrane bound concentric lamellar Ca^{2+} crystals within the principal cells of mosquito tubules Bradley et al. (1982), might interfere with measurement of Ca^{2+}.

Conclusions

Convincing evidence exists for the proton V-ATPase as the major source for generating the inwardly directed (lumen to cell) electrochemical proton gradient across the apical membrane of the principal cells in insect tubules (Maddrell and O'Donnell, 1992; Leyssens et al., 1993; Wessing, 1993; Zhang et al., 1994; O'Donnell and Maddrell, 1995; O'Donnell et al., 1996). Evidence for the V-ATPase has been presented in *A. aegypti* Malpighian tubules, as bafilomycin increased transepithelial resistance and depolarized membrane voltages (Pannabecker et al., 1993). The vertebrate Na^{+}/K^{+} ATPase provides some insight into how ATPase pumps are regulated. Specifically, neuroendocrine factors affect the phosphorylation state of the renal Na+/K+ ATPase (Aperia et al., 1994). Phosphorylation has also been suggested in the regulation of the vertebrate proton V-ATPase. The protein kinase C agonist, 4beta, 9alpha, 12beta, 13alpha, 20-pentahydroxytiglia-1,6-dien-3-one 12beta-myristate 13-acetate, 12-O-tetradecanoylphorbol 13-acetate (TPA), enhanced bafilomycin sensitive proton extrusion from mouse macrophages Nordstrom et al. (1994), and from human neutrophils (Nanda and Grinstein, 1991). Similar evidence has also been presented suggesting that protein kinase A and C are important in regulating the vertebrate renal Na^{+}/proton antiporter (Weinman and Shenolikar, 1986; Weinman et al., 1988).

Recent studies have suggested that unfacilitated water transport across epithelial cells can be influenced by cAMP mediated endocrine factors. Aquaporins are a family of transmembrane proteins that have been identified in the vertebrate kidney and function as molecular water channels (Chrispeels and Agre, 1994; Terris et al., 1995; Agre et al., 1995). Evidence suggests that vasopressin regulates these water channels in the kidney and that they are influenced by cAMP dependent phosphorylation (Kuwahara et al., 1995). In insects, evidence for aquaporins has been presented in *D. melanogaster* Malpighian tubules. Dow et al. (1995), presented immunocytochemical data identifying aquaporins on the basolateral surface of tubule stellate cells. A polymerase chain reaction (PCR) product taken from tubule mRNA contained a conserved aquaporin sequence. These data suggest

an alternate pathway for an endocrine regulated, unfacilitated water transport in insect tubules. These data suggest that neuroendocrine regulation of vertebrate transporting epithelia are mediated through changes in the phosphorylation state of these transporters. The insect Malpighian tubule proton V-ATPase-cation/proton antiporter complex could be influenced by neuroendocrine factors in a similar manner.

The apical membrane V-ATPase proton pump in insects is probably directly stimulated by cAMP dependent endocrine factors (Maddrell and O'Donnell 1992; O'Donnell et al., 1996), such as the diuresins. Activation of the apical V-ATPase that is complexed with the cation/proton antiporter(s) leads to an increase in cation movement across the tubule via active transport processes. In order to sustain this process, appropriate counter-anion movement is necessary. The leucokinins, that increase passive Cl^- permeability across the tubules (O'Donnell et al., 1996), may enable rapid anion response and may have an important role in regulating counter-anion flow during transepithelial fluid movement. Cation and anion movement across the epithelium are regulated by distinct processes (O'Donnell et al., 1996) and require different endocrine factors, probably operating through diverse cellular signal transduction pathways. Both aspects of this process must function in a coordinated manner to enable fluid movement across the tubules, leading to the formation of the primary urine.

The *C. salinarius* diuresin stimulated tubule cAMP and the mosquito leucokinins, in general, stimulated IP_3. The diuresins increased Na^+ conductance by influencing the activities of Na^+ channels and/or the $Na^+/K^+/2Cl^-$ cotransporter at the basolateral membrane. These changes in Na^+ conductance have also been induced with the application of DB cAMP in vitro (Hegarty et al., 1991). Diuresins alter the Na^+/K^+ ratio within the cell and increase monovalent cation transepithelial transport and fluid movement. The leucokinins appear to function by altering Cl^- conductance at paracellular junctions via a basolateral membrane related mechanism (Pannabecker et al., 1993), leading to increased transepithelial transport of monovalent cations. This study provides evidence in support of a Ca^{2+} dependent leucokinin stimulation of *A. aegypti* Malpighian tubules. The data demonstrated that the addition of leucokinins to isolated tubules lead to increased tubule IP_3, probably through a receptor mediated activation of the polyphosphoinositide pathway. Increases in cellular IP_3 have been associated with transient increases in cellular Ca^{2+} through the release of intracellular IP_3-sensitive Ca^{2+} stores (Berridge et al., 1983; Sekar and Hokin, 1986). The leucokinins increase transepithelial Cl^- conductance across mosquito Malpighian tubules (Pannabecker et al., 1993). This movement of Cl^- may

function as a counter anion to cation flow resulting in fluid movement and production of the primary urine.

As shown here, putative diuretic factors from two families of insect neuropeptides influence Malpighian tubule function. The *C. salinarius* diuresin and mosquito leucokinins stimulated mosquito tubule intracellular second messengers and increased urine production in vivo. Immunoneutralization experiments using antibodies raised against these neuropeptides suggests that they are involved in stimulating urine production.

APPENDIX A

Additional Methods Urine Production Bioassay

Urine production bioassay

Injection precision experiments. Injection precision was evaluated in mosquitoes (Table A.1) prior to initiating the urine production bioassays. Intact mosquitoes were injected with 0.5 μl radiolabeled water in saline, urine was collected for 30 minutes and radioactivity was measured. Comparison of total injected dpms for each mosquito with the mean total count dpms (TC) from injections applied under oil resulted in an overall difference of 5.4 %. Coefficient of variation between individual injections based on these data was 7.5 %. These data demonstrate that minimum variation exists between individual injections and from total count injections.

Table A.1. Evaluation of injection precision in urine production bioassay experiments.

Mosquito (dpm)	Urine Collected (dpm)	Total Injected (dpm)	Percent From TC
40723	4426	45149	10.7
50393	5475	55868	9.4
48391	2414	50805	0.4
43609	4375	47984	5.1
50629	2788	53417	5.2
46621	4860	51633	2.0
47143	3442	50585	0.0
40717	4433	45150	10.7

TC (total counts) representing injections applied under oil. TC was 50596 dpm (n = 3).

Injection volume and baseline urine production. Figure A.1 represents the results from experiments comparing two different injection volumes on the effect of total urine production. Injection volumes of 1.0 μl appeared to increase, but not significantly, baseline urine production compared to mosquitoes injected with 0.5 μl. All urine production bioassays were conducted using 0.5 μl injections to minimize injection volume induced increases in urine production.

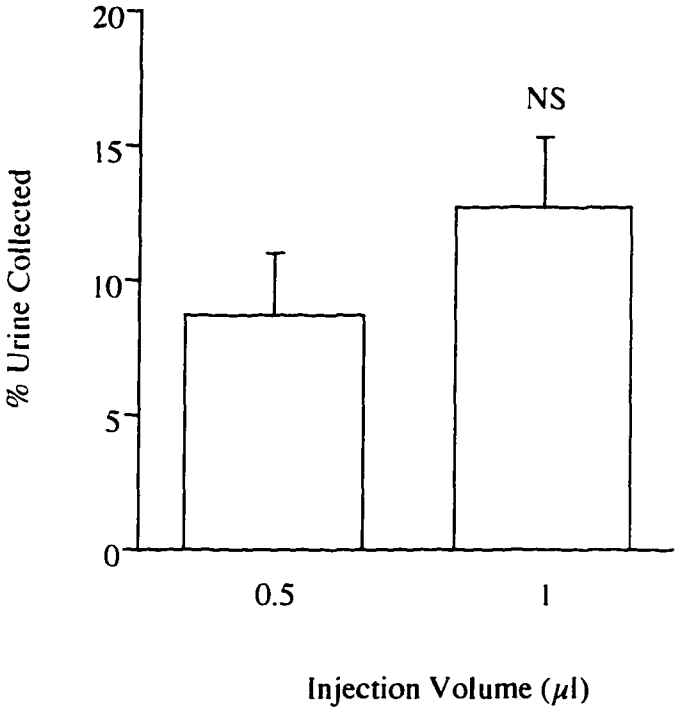

Fig. A.1. Injection volume and total urine production in intact mosquitoes. Values are means ± SE, 6 mosquitoes per bar, 30 minute incubation following injection, NS = not significant.

APPENDIX B

Additional Methods Second Messengers Assays

Experiments for cAMP determination

Extraction efficiency for cAMP. Tissue extraction efficiency for cAMP was determined in experiments using radiolabeled cAMP ([5',8-^{3}H] adenosine 3',5'-cyclic phosphate, ammonium salt, specific activity 143 mCi/mg) with Malpighian tubule extracts. Figure B.1 shows the results from an experiment in which differences in extraction solvent efficiencies were measured. From these data it can be concluded that ethanol plus concentrated HCL (60:1) resulted in optimal extraction efficiency of 90.8 ± 1.7 % for cAMP from Malpighian tubule tissue.

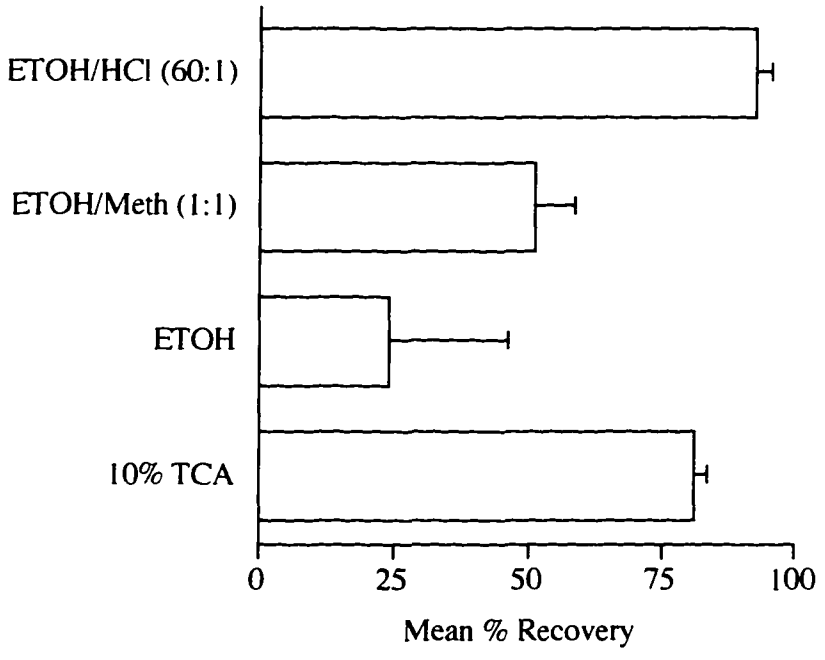

Fig. B.1. Tissue extraction efficiency for cAMP from Malpighian tubules using different extraction solvents. Each bar represents 4 separate extraction trials, (ETOH = ethanol, Meth = Methanol, HCl = hydrochloric acid, TCA = trichloroacetic acid), values are means ± SE.

Recovery of cAMP from anion exchange chromatography. Anion exchange chromatography was used to isolate endogenous cAMP from tubule extracts. Figure B.2 represents the results from anion exchange chromatography of cAMP using Dowex 1 X 8, 100-200 mesh converted to the formate form (Bio-Rad, Richmond, CA). Samples were diluted in water, applied to the mini column and eluted with 2 M/L formic acid. Total recovery from all fractions for radiolabeled cAMP in Malpighian tubule extracts was 90.6 %.

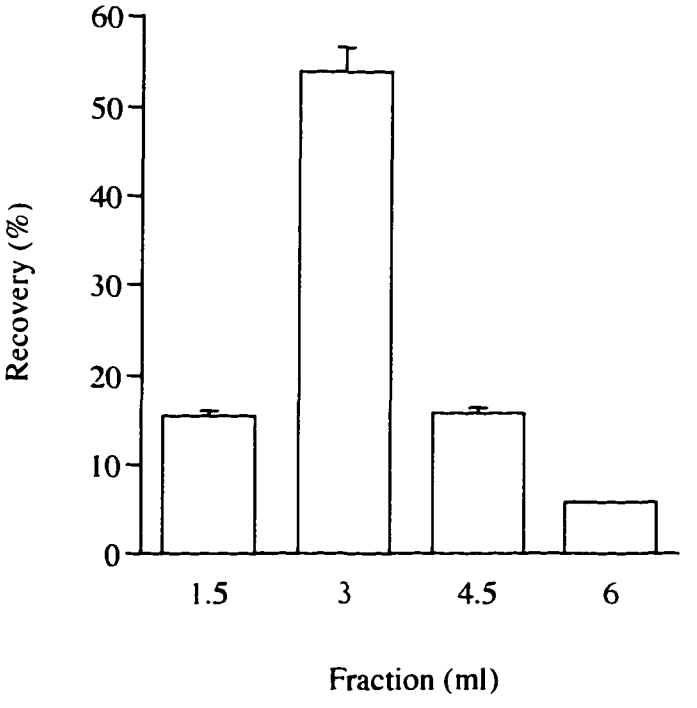

Fig. B.2. Recovery of radiolabeled cAMP from anion exchange chromatography. Values are means ± SE, 3 anion exchange mini columns per bar.

Comparing two different ELISA systems. The Kingan ELISA cAMP was compared to a commercially available ELISA cAMP kit from Gibco BRL. Malpighian tubules, stimulated with forskolin, were extracted, pooled and assayed in both ELISAs. Intracellular cAMP increased in Malpighian tubules treated with 100 μM/L of forskolin with 5 X 10^{-4} M/L IBMX. As shown in figure B.3, the Gibco BRL ELISA system and the Kingan ELISA gave similar estimates for intracellular cAMP. These data demonstrate

that both ELISA methods are measuring similar amounts of endogenous cAMP from the same sample. This suggests that the Kingen ELISA provided an estimate of intracellular cAMP concentrations in mosquito tubules similar to that of a commercial kit.

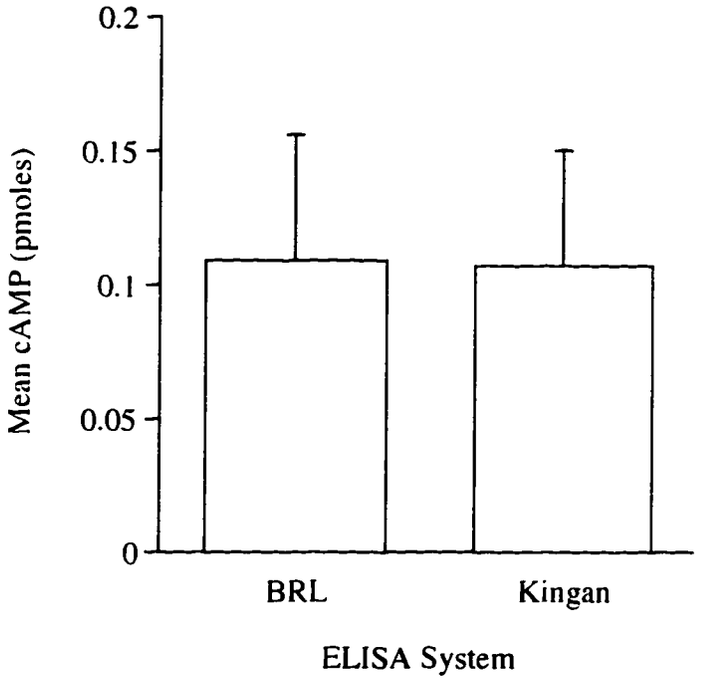

Fig. B.3. Intracellular cAMP concentrations from tubule extracts measured with different ELISAs. Gibco BRL = 0.108 ± 0.046 pmoles cAMP, compared to the modified Kingan ELISA = 0.106 ± 0.043 pmoles cAMP. Each bar represents 9 separate samplings from pooled extract of Malpighian tubules, values are means ± SE.

Time course stimulation of tubule cAMP with forskolin. Malpighian tubules were stimulated for 2-30 min with 100 μM/L forskolin with 5 X 10^{-4} M/L of IBMX and assayed for intracellular cAMP. Figure B.4 shows the results of these timed stimulations. Peak stimulation of cAMP in tubules occurred at 15 and 30 minutes.

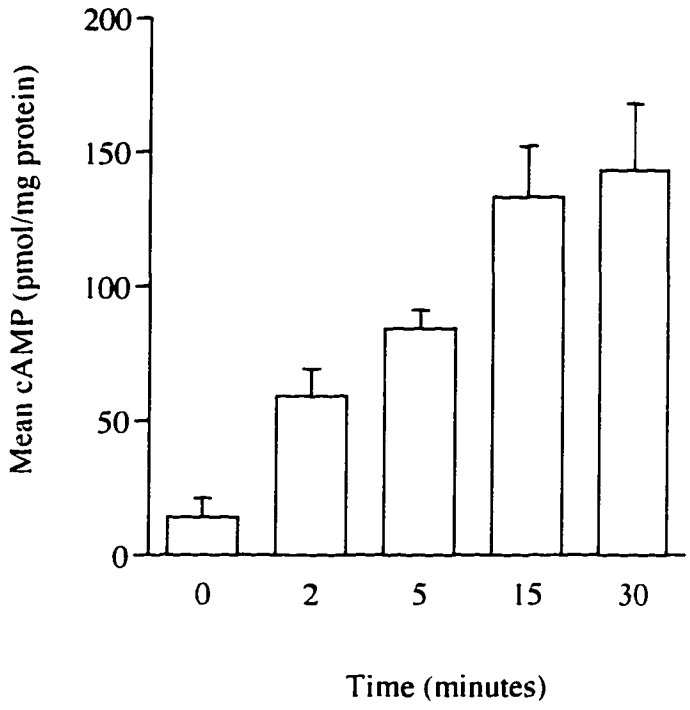

Fig. B.4. Timed forskolin stimulation of Malpighian tubule intracellular cAMP. Values are means ± SE, 6 mosquitoes per bar.

Experiments for IP3 determination

Extraction efficiency for IP3. Different extraction solvents were tested to optimize the extraction of intracellular IP3 from Malpighian tubules. Figure B.5 shows data from experiments in which radiolabeled IP3 was added to Malpighian tubule tissue prior to extraction. Optimum extraction efficiency was achieved with several of the solvents tested; only 15% trichloroacetic acid washed in freon tri-n-octylamine resulted in extracts free of compounds that interfered with the IP3 binding protein assay. The TCA/freon tri-n-octylamine solvent system was used in all tubule in vitro stimulation experiments which examined tubule IP3 responses.

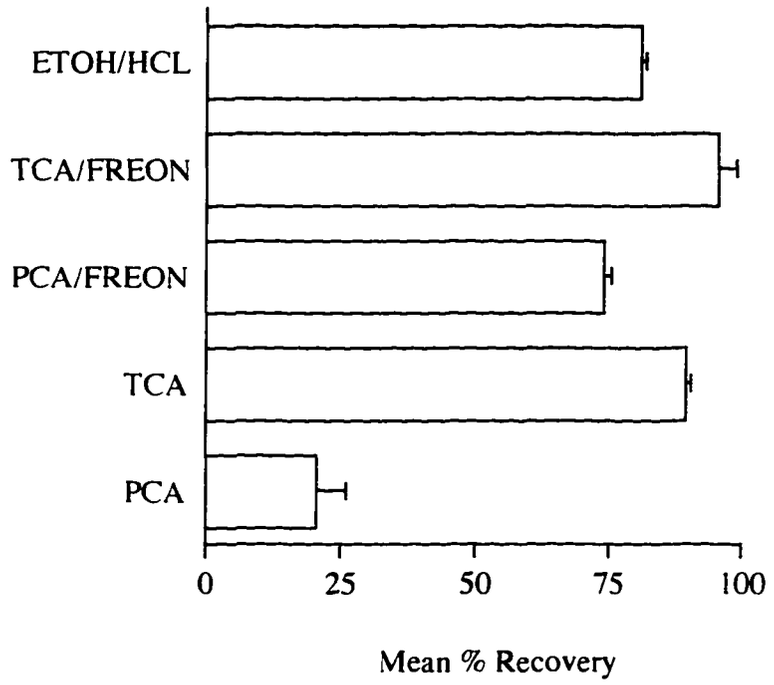

Fig. B.5. Tissue extraction efficiency for IP_3 from Malpighian tubules using different extraction solvents. Each bar represents 3 separate extraction trials, (ETOH/HCL = ethanol and hydrochloric acid (60:1), TCA/FREON = 15% trichloroacetic acid washed with freon tri-n-octylamine (1:1), PCA/FREON = 20% perchloric acid washed with freon tri-n-octylamine (1:1), TCA = 15% trichloroacetic acid, PCA = 20% perchloric acid), values are means ± SE.

Recovery of IP3 from anion exchange chromatography. Anion exchange chromatography was use to isolate IP_3 in order to determine if compounds were present in Malpighian tubule extracts that interfere with the IP_3 binding protein assay. Figure B.6 represents the recovery of radiolabeled IP_3 in Malpighian tubule extracts from anion exchange mini columns using Dowex 1 X 8, mesh 400 converted to the formate form . Samples were diluted in water, applied to the column and eluted with 0.8 M/L ammonium formate in 0.1 M/L formic acid. Total recovery from mini colums, for all fractions was equal to 85.1%.

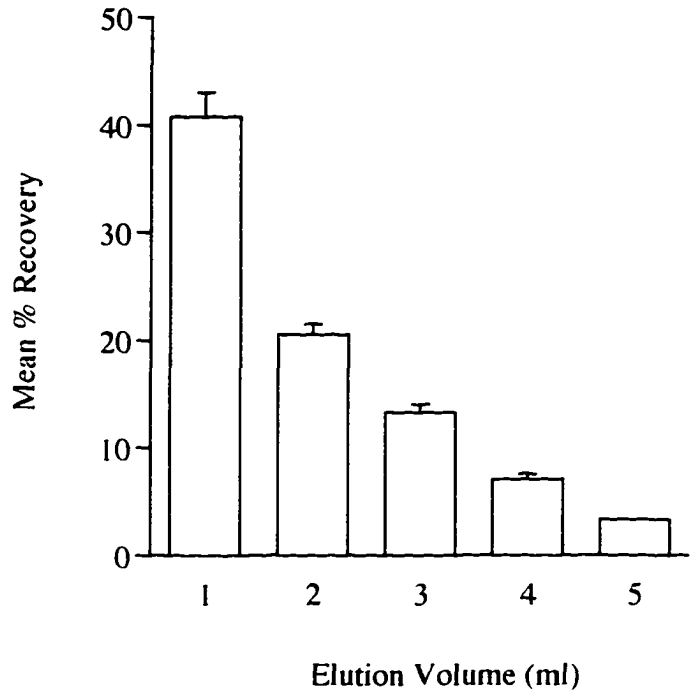

Fig. B.6. Recovery of radiolabeled IP3 from anion exchange chromatography. Values are means ± SE, 3 columns per bar, 5 ml fraction error bar is too small to appear on the graph.

REFERENCES

Agre P., Brown D. and Nielsen S. (1995) Aquaporin water channels: unanswered questions and unresolved controversies. *Curr. Opin. Cell Biol.* **4**, 472-483.

Aperia A., Holtback U, Syren M. L., Svensson L. B., Fryckstedt J. and Greengard P. (1994) Activation /deactivation of renal Na^+,K^+-ATPase: a final common pathway for regulation on natriuresis. *FASEB J.* **8**, 436-439.

Audsley N., Kay I., Hayes T. K. and Coast G. M. (1995) Cross reactivity studies of CRF-related peptides on insect Malpighian tubules. *Comp. Biochem. Physiol.* **110A**, 87-93.

Baumgold J., Paek R. and Fiskum G. (1992) Calcium independence of phosphoinositide hydrolysis-induced increase in cyclic AMP accumulation in SK-N-SH human neuroblastoma cells. *J. Neurochem.* **58**, 1754-1759.

Berridge M. J. (1981) Electrophysiologicial evidence for the existence of separate receptor mechanisms mediating the action of 5-hydroxytryptamine. *Mol. Cell. Endocrinol.* **23**, 91-104.

Berridge M. J. and Heslop J. P. (1981) Separate 5-hydroxytryptamine receptors on the salivary gland of the blowfly are linked to the generation of either cyclic adenosine 3',5'-monophosphate or calcium signals. *Br. J. Pharmac.* **73**, 729-738.

Berridge M. J., Dawson R. M. C., Downes P. C., Heslop J. P. and Irvine R. F. (1983) Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. *Biochem. J.* **212**, 473-482.

Bradley T. J., Stuart A. M. and Satir P. (1982) The ultrastructure of the larval Malpighian tubules of a saline-water mosquito. *Tissue Cell* **14**, 759-773.

Chrispeels M. J. and Agre P. (1994) Aquaporins: water channel proteins of plant and animal cells. *Trends Biochem. Sci.* **19**, 421-425.

Clark T. M. (1994) Ph.D. Dissertation, University of California, Irvine. Hormonal control of Malpighian tubules in larval *Aedes aegypti*.

Clark T. M. and Bradley T. J. (1996) Stimulation of Malpighian tubules from larval Aedes aegypti by secretagogues. *J. Insect Physiol.* **42**, 593-602.

Clottens F. L., Holman G. M., Coast G. M., Totty N. F., Hayes T. K., Kay I., Mallet A. I., Wright M. S., Chung J. S., Truong O. and Bull D. L. (1994) Isolation and characterization of a diuretic peptide common to the house fly and stable fly. *Peptides* **15**, 971-979.

Coast G. M. and Kay I. (1994) The effects of *Acheta* diuretic peptide on isolated Malpighian tubules from the house cricket *Acheta domesticus*. *J. Exp. Biol.* **187**, 225-243.

Coast G. M., Holman G. M. and Nachman R. J. (1990) The diuretic activity of a series of cephalomyotropic neuropeptides, the achetakinins, on isolated Malpighian tubules of the house cricket, *Acheta domesticus*. *J. Insect Physiol.* **36**, 481-488.

Coast G. M., Hayes T. K., Kay I. and Chung J. (1992) Effect of Manduca sexta diuretic hormone and related peptides on isolated Malpighian tubules of the house cricket *Acheta domesticus* (L.). *J. Exp. Biol.* **162**, 331-338.

Coast G. M., Rayne R. C., Hayes T. K., Mallet A. I., Thompson K. S. J. and Bacon J. P. (1993) A comparison of the effects of two putative diuretic hormones from *Locusta migratoria* on isolated locust Malpighian tubules. *J. Exp. Biol.* **175**, 1-14.

Davies S. A., Huesmann G. R., Maddrell S. H. P., O'Donnell M. J., Skaer N. J. V., Dow J. A. T. and Tublitz N. J. (1995) CAP2b, a cardioacceleratory peptide, is present in Drosophila and stimulates tubule fluid secretion via cGMP. *Am. J. Physiol.* **269**, R1321-R1326.

Dow J. A. T., Kelly D. C., Davies S. A., Maddrell S. H. P. and Brown D. (1995) A novel member of the major intrinsic protein family in *Drosophila*: are aquaporins involved in insect Malpighian (renal) tubule fluid secretion? *J. Physiol.* **489**, 110P-111P.

Fogg K., Anstee J. and Hyde D. (1990) Effects of corpora cardiaca extract on intracellular second messenger levels in Malpighian tubules of *Locusta migratoria* L. *J. Insect Physiol.* **36**, 383-389.

Hayes T. K., Pannabecker T. L., Hinckley D. J., Holman G. M., Nachman R. J., Petzel D. H. and Beyenbach K. W. (1989) Leucokinins, a new family of ion transport stimulators and inhibitors in insect Malpighian tubules. *Life Sci.* **44**, 1259-1266.

Hegarty J., Zhang B., Pannabecker T., Petzel D., Baustian M. and Beyenbach K. W. (1991) Dibutyryl cAMP activates bumetanide-sensitive electrolyte transport in Malpighian tubules. *Am. J. Physiol.* **261**, C521-C529.

Kataoka H., Troetschler R., Li J., Kramer S., Carney R. and Schooley D. (1989) Isolation and identification of a diuretic hormone from the tobacco hornworm, *Manduca sexta*. *Proc. Natl. Acad. Sci.* **86**, 2976-2980.

Kuwahara M., Fushimi K., Terada Y., Bai L., Marumo F. and Sasaki S. (1995) cAMP-dependent phosphorylation stimulates water permeability of aquaporin-collecting duct

water channel protein expressed in Xenopus oocytes. *J. Biol. Chem.* **270**, 10384-10387.

Lange A. B., Orchard I. and Barrett F. M. (1989) Changes in haemolymph serotonin levels associated with feeding in the blood-sucking bug, *Rhodnius prolixus*. *J. Insect Physiol.* **35**, 393-399.

Leyssens A., Zhang S. L., Van Kerkhove E. and Steels P. (1993) Both dinitrophenol and Ba^{2+} reduce KCl and fluid secretion in Malpighian tubules of *Formica ployctena*: and the role of the apical H^+ and K^+ concentration gradient. *J. Insect Physiol.* **39**, 1061-1073.

Leyssens A., Kerkhove E. V., Zhang S. L., Weltens R. and Steels P. (1993) Measurement of intracellular and lumenal K^+ concentrations in a Malpighian tubule (Formica). Estimate of basal and luminal electrochemical K^+ gradients. *J. Insect Physiol.* **39**, 945-958.

Maddrell S. H. P. and O'Donnel M. J. (1992) Insect Malpighian tubules: V-ATPase action in ion and fluid transport. *J. Exp. Biol.* **172**, 417-429.

Maddrell S. H. P., Herman W. S., Mooney R. L. and Overton J. A. (1991) 5-Hydroxytryptamine: a second diuretic hormone in *Rhodnius prolixus*. *J. Exp. Biol.* **156**, 557-566.

Nanda A. and Grinstein S. (1991) Protein kinase C activates an H^+ (equivalent) conductance in the plasma membrane of human neurtophils. *Proc. Natl. Acad. Sci.* **88**, 10816-10820.

Nordstrom T., Grinstein S., Brisseau G. F., Manolson M. F. and Rotstein O. D. (1994) Protein kinase C activation accelerates proton extrusion by vacuolar-type (H^+)-ATPase in murine peritoneal macrophages. *FEBS Lett.* **350**, 82-86.

Novak M. G., Ribeiro J. M. C. and Hildebrand J. G. (1995) 5-hydroxytryptamine in the salivary glands of adult female Aedes aegypti and its role in regulation of salivation. *J. Exp. Biol.* **198**, 167-174.

O'Donnell M. J. and Maddrell S. H. P. (1995) Fluid reabsorption and ion transport by the lower Malpighian tubules of adult female Drosophila. *J. Exp. Biol.* **198**, 1647-1653.

O'Donnell M. J., Dow J. A. T., Huesmann G. R., Tublitz N. J. and Maddrell S. H. P. (1996) Separate control of anion and cation transport in Malpighian tubules of *Drosophila melanogaster*. *J. Exp. Biol.* **199**, 1163-1175.

Pannabecker T. L. and Beyenbach K. W. (1993) Time-dependent mechanisms of action of bafilomycin in Malpighian tubules. *Fed Proc.* **7**, A580.

Pannabecker T. L., Hayes T. K. and Beyenbach K. W. (1993) Regulation of epithelial shunt conductance by the peptide leucokinin. *J. Membr. Biol.* **132**, 63-76.

Patel M., Hayes T. K. and Coast G. M. (1995) Evidence for the hormonal function of a CRF-related diuretic peptide (*Locusta*-DP) in *Locusta migratoria*. *J. Exp. Biol.* **198**, 793-804.

Petzel D. (1985) Preliminary isolation of mosquito natriuretic factor. *Am. J. Physiol.* **249**, R379-R386.

Saudou F., Boschert U., Amlaiky N., Plassat J. L. and Hen R. (1992) A family of Drosophila serotonin receptors with distinct intracellular signalling properties and exptression patterns. *EMBO J.* **11**, 7-17.

Sekar M. C. and Hokin L. E. (1986) The role of phosphoinositides in signal transduction. *J. Membrane Biol.* **89**, 193-210.

Terris J., Ecelbarger C. A., Marples D., Knepper M. A. and Nielsen S. (1995) Distribution of aquaporin-4 water channel expression within rat kidney. *Am. J. Physiol.* **269**, F775-F785.

Thompson K. S. J., Rayne R. C., Gibbon C. R., May S. T., Patel M., Coast G. M. and Bacon J. P. (1995) Cellular colocalization of diuretic peptides in locusts: a potent control mechanism. *Peptides* **16**, 95-104.

Tublitz N. J. (1988) Insect cardioactive neuropeptides: peptidergic modulation of the intrinsic ryhthm of an insect heart is mediated by inositol 1,4,5-trisphosphate. *J. Neurosci.* **8**, 4394-4399.

Veenstra J. (1988) Effects of 5-hydroxytryptiamine on the Malpighian tubules of *Aedes aegypti*. *J. Insect Physiol.* **34**, 299-304.

Weinman E. J. and Shenolikar S. (1986) Protein kinase C ativates the renal apical membrane Na+/H+ exchanger. *J. Membr. Biol.* **93**, 133-139.

Weinman E. J. Dubinsky W. P. and Shenolikar S. (1988) Reconstitution of cAMP-dependent protein-kinase-regulated renal Na^+-H^+ exchanger. *J. Membr. Biol.* **101**, 11-18.

Wessing A. (1993) Effects of bafilomycin A1 and amiloride on the apical potassium and proton gradients in Drosophila Malpighian tubules studied by X-ray microanalysis and microelectrode measurements. *J. Comp. Physiol.* **163**, 452-465.

Wessing A., Bertam G. and Zierold K. (1993) Effects of bafilomycin A1 and amiloride on the apical potassium and proton gradients in *Drosophila* Malpighian tubules studied by

X-ray microanalysis and microelectrode measurements. *J. Comp. Physiol. B.* **163**, 452-462.

Williams J. and Beyenbach K. W. (1983) Differential effects of secretagogues on Na and K secretion in the Malpighian tubules of *Aedes aegypti* (L.). *J. Comp. Physiol.* **149**, 511-517.

Zhang S. L., Leyssens A., Van Kerkhove E., Weltens R., Driessche W. V. and Steels P. (1994) Electrophysiological evidence for the presence of an apical H^+-ATPase in Malpighian tubules of Formica polyctena: intracellular and luminal pH measurements. *Pflugers Arch* **426**, 288-295.