LIMBURGS UNIVERSITAIR CENTRUM

PURIFICATION, CHARACTERIZATION AND MODE OF ACTION OF ENDOGENOUS NEUROENDOCRINE FACTORS IN THE FOREST ANT, *FORMICA POLYCTENA*

Promotoren :
Prof. dr. E. Van Kerkhove (L.U.C)
Prof. dr. P. Steels (L.U.C)

Co-promotor : Prof. dr. L. Schoofs (K.U.Leuven)

Faculteit Wetenschappen

Bart Laenen, 1998

Eindwerk aangeboden tot het behalen van de graad van Doctor in de Wetenschappen.

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LIST OF ABBREVIATIONS

Acd-DP	*Acheta domesticus* diuretic peptide
AKH	adipokinetic hormones
ant-eq/µl	ant equivalents / µl
Apm-ADF	*Apis mellifera* antidiuretic factor
Apm-DF	*Apis mellifera*diuretic factor
AU	absorption unit
Baf-A1	bafilomycin
BCA	bicinchoninic acid
BSA	bovine serum albumin
CAP_{2b}	cardioaccelatory peptide
CHE	crude hemolymph extract
cNOS	constitutive NOS
cpt-cAMP	chlorophenylthio-cyclic AMP
CRF	corticotropin releasing factor
DMSO	dimethyl sulfoxide
DNP	dinitrophenol
EH	eclosion hormone
ELISA	enzyme linked immunosorbent assay
ETH	ecdysiotropic hormone
Fop-cGP	*Formica polyctena* cAMP generating peptide
Fop-DP	*Formica polyctena* diuretic peptide
Fop-DF	*Formica polyctena* diuretic factor
Fop-TK I	*Formica polyctena* tachykinin I
Fop-TK II	*Formica polyctena* tachykinin II
FopADF	*Formica polyctena* antidiuretic factor
HPLC	high performance liquid chromatography
IBMX	3-isobutyl-1-methylxanthine
iNOS	inducible NOS
ITP	Ion transport peptide
Lom-DH	*Locusta migratoria* diuretic hormone
MALDI-TOF	Micromass TofSpec Matrix Assisted Laser Desorption-
Mas-DH	*Manduca sexta*diuretic hormone

MS	mass spectroscopy
MT	Malpighian tubules
NEM	N-ethylmalmeimide
NOS	nitric oxide synthetase
PB	Phosphate buffer
pmt	photomultiplier tube
PTH	Phthaldialdehyde
PTTH	prothoracicotropic hormones
RP-HPLC	reversed-phase high performance liquid chromatography
SEM	standard error of the mean
SNAP	S-nitroso-N-acetylpenicillamine
SNP	sodiumnitroprusside
TFA	trifluoroacetic acid
	time of Flight
V_{ap}	apical membrane potential
V_{bl}	basolateral membrane potential
V_{te}	transepithelial membrane potential

GENERAL INTRODUCTION

I. STATE OF THE ART

Morphology of the Malpighian tubules

" Inter alimenti excrementique receptacula, hoc est, in angustatur entriculi parte ...,
tenuia quaedam & varicose emergunt vasa... '
Malpighi, De Bombyce, to the Royal Society of London, 1669.

In this quotation Malpighi describes for the first time the "drawn-out and varicose vessels" that were later to be named in his honor. In the 1669 paper Malpighi accurately describes the anatomy of the Malpighian tubules of the silkworm, *Bombyx mori*.
Today Malpighian tubules of many species have been described. We now know that they are extremely variable in number, length, shape, histology and position in the body. In general, they can be described as a series of blind ending tubules which lie in the body cavity (Wigglesworth, 1931), and have rather free contact with the haemolymph. The distal end of each tubule is closed. It may lie free in the body cavity, or be buried in the walls of the rectum forming a cryptonephridial system (Cochran, 1973). Proximally, the tubules are attached to the gut in the region of the midgut-hindgut junction. They empty into the gut, but the morphology of the tubule-hindgut connection varies between species. For example in *Drosophila* the 4 tubules pairwise join a common ureter, which opens into the hindgut (Skaer, 1996), while in *Acheta* there are more than 100 tubules which end in a common ampulla. In the forest ant, *Formica polyctena*, 12 - 18 tubules which are 2-3 mm long, empty directly into a posterior midgut ampullar region at the beginning of the hindgut (Garayoa et al., 1992) (Fig. 0.1). The tubules may move in the body cavity in response to body movements or as a result of contractions by the muscles which are wound round them in a spiral way (Cochran, 1973).

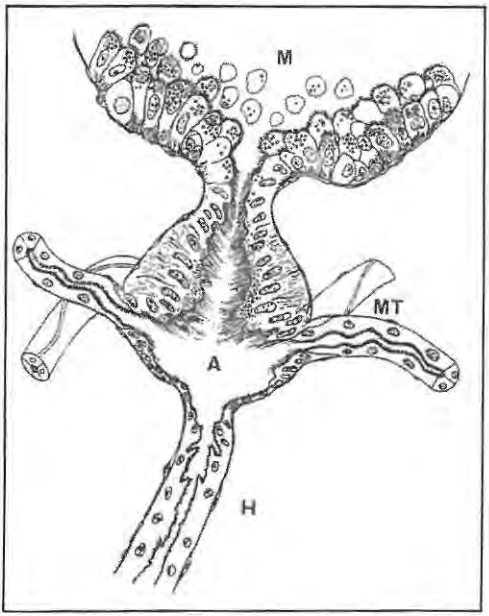

Figure 0.1 Scheme of *Formica polyctena* Malpighian tubules (MT) draining into the gut through a specialized posterior midgut ampullar region.M, midgut; H, hindgut; A, ampulla. (Reproduced from Garayoa *et al.* , 1992)

The excretory system in insects

Today the excretory system of insects is known to consist of two parts: the Malpighian tubules which secrete the primary urine and carry out the first modifications in composition and the ileum and especially the rectum in which the fine regulation of the urine composition is achieved and which delivers the final urine.

In the beginning of the nineteenth century Herold (1815) recognized for the first time that the **Malpighian tubules** are the principal excretory organ of insects (see Wigglesworth 1972 for accounts of history) (Wigglesworth, 1972). Primary urine formation in the Malpighian tubule lumen is achieved by active ion transport (mostly KCl), followed by water. A **model**, summarizing the mechanism involved in active NaCl and / or KCl transport is shown in Fig. 0.2.

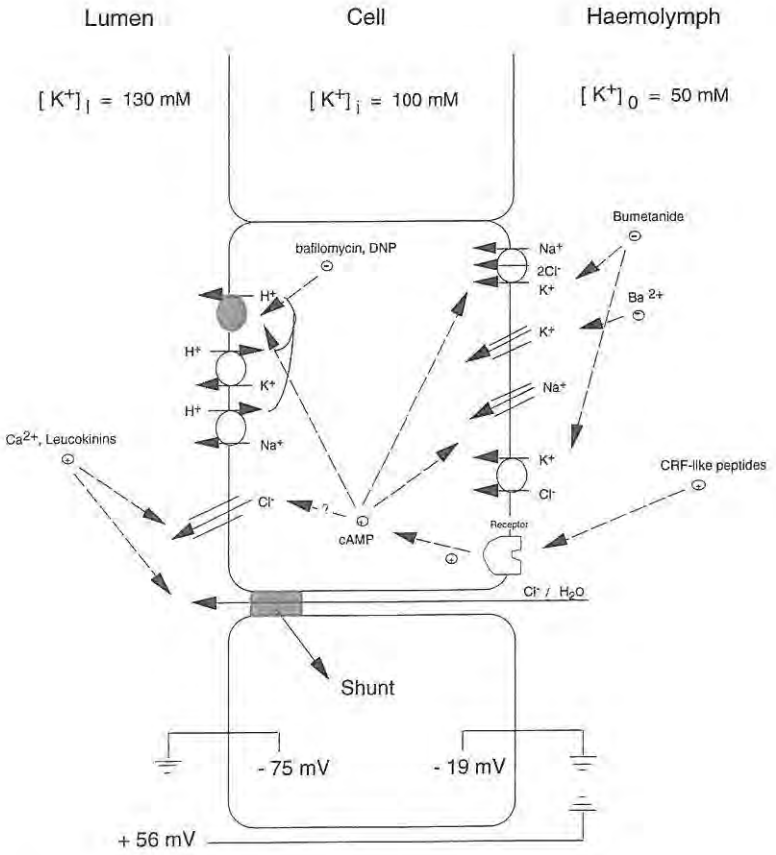

Figure 0.2 Model for potassium and sodium transport across the Malpighian tubule cells of insects. Hatched circles denote ATP-requiring processes. Open circles indicate gradient - dependent and secondary active transporters.

In this model sodium and / or potassium are transported against a steep electrochemical gradient, i.e. both a concentration and an electrical gradient (Leyssens et al., 1992). As the cation transport is energy dependent it must necessarily pass through the cells. At the basolateral side, Na^+ and K^+ enter the cell both via ion channels and electroneutral

transporters such as the bumetanide-sensitive Na^+ / K^+ / $2Cl^-$ and K^+/Cl^- cotransporters (Leyssens et al., 1994). The active step resides in the apical membrane: an apical proton-motive force, generated by a bafilomycin-sensitive V-type H^+ ATPase (Garayoa et al., 1995),(Weltens et al., 1992),(Zhang et al., 1994) is used to energize two discrete secondary active antiporters extruding K^+ and / or Na^+ in exchange for H^+ (Pannabecker, 1995). The active translocation of K^+ and / or Na^+ from hemolymph to lumen is followed by passive transport of Cl^- and water across the epithelium (Dijkstra et al., 1994).

Immunocytochemistry and electrophysiology were the major tools to proof the presence of the different transport mechanisms. For the ant, the sensitivity of the active ion transport to Ba^{2+} suggested the presence of basolateral K^+ ion channels (Leyssens et al., 1992). From similar experiments it could be concluded that for a low potassium concentration in the bath, bumetanide sensitive transport mechanisms are involved (Leyssens et al., 1994). Immunocytochemistry confirmed the presence of the V-type H^+ ATPase at the apical membrane (Garayoa et al., 1995) as well as the presence of the K^+/H^+ and the Na^+/H^+ antiporters (Harvey and Ehrenfeld, 1988).

The epithelium is relatively leaky so that other ions, essential substances and potentially toxic organic compounds pass into the primary secreted fluid. The **primary urine** produced by the Malpighian tubules is subject to considerable modification before it leaves the body with the excreta. Some reabsorption of water and ions, as well as a lowering of the pH, may occur in the proximal portion of the tubule itself (Haley and O'Donnell, 1997),(Kim and Spring, 1992),(Spring and Clark, 1990). Subsequently, the anterior region of the hindgut removes some substances, including water, and continues the acidification process (Cochran, 1973), (Spring, 1990). The resulting fluid enters the **rectum** which is responsible for the major modifications which are important both for excretion of waste substances and for osmoregulation. It modifies the primary urine by removing ions, water and other useful small molecules such as amino acids and sugars.

Essential substrates such as amino acids are reabsorbed luminally via Na^+-cotransporters, using a cell inward directed Na^+-gradient, sustained by the Na^+/K^+-pump which is localized along the basolateral plasma membrane (Phillips, 1981). However, active reabsorption of Cl^- at the apical membrane, via a cAMP sensitive mechanism (Hanrahan and Phillips, 1983) is the predominant transport process in insect recta. The net K^+-reabsorption from the lumen side, is largely passive, being electrically coupled to the Cl^--transport. Furthermore the rectum completes the acidification of the excreta which is necessary to precipitate uric acid, via an apical V-type H^+-ATPase (Thomson and Phillips,

1992). In conclusion, the rectum is a crucial tissue for osmoregulation in response to the physiological needs of the insect as mediated through the hormonal system.

Endocrine regulation of the excretory system in insects

Since insects are exposed to a varying environment and to changes challenging the volume and composition of extracellular and intracellular fluid compartments (Beyenbach, 1992),(1992),(Zeiske, 1992), appropriate and fast regulation of the Malpighian tubule - rectum excretory system is required. Not suprisingly, both rectum and Malpighian tubules are under endocrine hormonal control.

a) **Rectum**: determines the final urine composition.

Diuretic and antidiuretic hormones active on the **rectum** have been found in several insect species. **Diuretic** or neuroendocrine factors that decrease water reabsorption in the rectum of insects have only been reported for crude extracts made of brain, corpora cardiaca or suboesophageal ganglion (Proux, 1990). All of these studies were conducted on poorly characterized in vitro preparations under transient conditions, and as such it is unclear whether these putative diuretic hormones act on rectal ion transport, osmotic permeability or metabolism (Gäde et al., 1997). Amongst the **antidiuretic** factors, best characterized are the neuroparsins and the locust ion transport peptide (ITP), isolated from corpora cardiaca extracts of the migratory locust. These are polypeptides (7 - 9 kD) which stimulate the fluid reabsorption at the rectal level (Girardie and Fournier, 1993). This antidiuretic effect is directed towards the conservation of water, and seems to be mediated via an inositol 1,4,5-triphosphate (IP_3) regulated increase in intracellular calcium (Fournier, 1990) for the neuroparsins, and via cAMP as a second messenger for ITP (Phillips, 1982).

b) **Malpighian tubules**: determine the primary urine composition.

Diuretic factors active directly on the **Malpighian tubules** have also been described. Malpighian tubule secretion is stimulated by a variety of neuroendocrine factors that include both biogenic amines (notably 5-hydroxytryptamine, 5-HT) and peptides (Maddrell et al., 1991). Two classes of diuretic peptides have been characterized. The first family of diuretic peptides shows great structural relation to the vertebrate CRF (corticotropin releasing factor) / urotensin / sauvagine family of peptides and is therefore named the family of CRF-related diuretic peptides. The second family belongs to the myokinins.

The **CRF-related peptides** range in size from 30 to 46 amino acids and have an average sequence homology of 50%. Their mode of action appears to be relatively uniform across insect species and is mediated through an increase in intracellular cAMP (Audsley et al.,

1995). Diuretic factors, such as the CRF-related peptides, which increase the intracellular cAMP concentration, may regulate the salt transport at different points (see Fig. 0.2). In mosquitos for example an elevated cAMP level increases the conductance of cAMP sensitive Na^+-channels in the basolateral membrane (Beyenbach, 1995) and activates the bumetanide sensitive Na^+ / K^+ / 2 Cl^- - transporter (Pan et al., 1984). This increase of the basolateral membrane Na^+ conductance in response to cAMP explains the shift towards a sodium rich primary urine after a blood meal in hematophagous insects (Maddrell et al., 1991),(Petzel et al., 1985). In *Drosophila* tubules (O'Donnell et al., 1998) the increase in intracellular cAMP was also shown to stimulate the activity of the apical V-type H^+ ATPase and as a consequence to accelerate the cation transport by stimulating the Na^+ / H^+ and K^+ / H^+ exchange.

The second class of diuretic peptides or **myokinins** were initially isolated on the basis of their myotropic activity on the hindgut of *Leucophaea maderae* (Holman et al., 1991). They are small peptides (6-13 residues) with a highly conserved C-terminal pentapeptide sequence. The integrity of this C-terminal core sequence, is essential for their biological activity (Nachman et al., 1995). It has been proven that the kinins have an additive effect to cAMP stimulated tubules in *Locusta* (Coast, 1995) and *Drosophila* (O'Donnell et al., 1996), suggesting they act via a cAMP independent mechanism. The second messenger could be calcium: the diuretic effect was mimicked by calcium ionophores and by calcium mobilizing drugs such as thapsigargin (Coast, 1995). Moreover intracellular calcium was shown to increase in aequorin transfected stellate cells of *Drosophila* Malpighian tubules in the presence of the kinin (O'Donnell et al., 1998). Changes in the permeability of the tubules for the passive transport of Cl^- and water, could also affect the primary urine production in insects. An increase in Cl^- permeability will facilitate the rate with which Cl^- can follow the actively transported cations (see Fig. 0.2). As a result the fluid secretion rate will increase. A Ca^{2+} mediated increase in Cl^--conductance, has been demonstrated in *Drosophila melongaster* (O'Donnell et al., 1998) and *Aedes aegypti* (Wang et al., 1996) Malpighian tubules: it was triggered with leucokinins (O'Donnell et al., 1998),(Wang et al., 1996), members of the second class of diuretic peptides in insects, the myokinins.

Interestingly, kinins and CRF-related peptides act in a synergistic way. Best characterized is the **synergism** between locustakinin, a myokinin and *Locusta migratoria* diuretic hormone (Lom-DH), a CRF-related peptide, in Locust tubules (Coast, 1995). At low concentrations, the peptides co-operatively stimulate fluid secretion to a value higher than the sum of the values they would have reached if they had been applied separately. The

calcium mobilizing agent, thapsigargin, mimicked the synergistic behaviour of locustakinin, and the cAMP analogue 8-Br-cAMP that of Lom-DH. This synergistic effect of two stimulants acting via different second messengers resembles the synergistic effect of biogenic amines (octopamine and 5-HT) and cardioacceleratory peptides on the excitation of the heart of adult *Manduca sexta* (Prier et al., 1994). A first advantage of dual control consists in the amount of material needed to get an effect on the primary urine production. If a single peptide had to do the job, 50% of the store present in the storage cells would be needed to get a maximal response. Whereas only 2.5% of the total store was needed in the presence of the synergistic factor (Coast, 1995). Consequently less energy is required since peptide synthesis is metabolically expensive, especially for larger peptides like the CRF-related ones. A second advantage is related to improved tuning. A faster and finer regulation can be obtained with two peptides (Coast, 1996). The fluid secretion will be sensitive to small changes in circulating peptide concentrations.

The importance of a **dual control** of primary urine production with regard to peptide synthesis and fine regulation suggests that this mechanism might well be conserved in insects. This idea is supported by the fact that CRF-related peptides and myokinins have been identified for all the insect species studied so far. There are indications that this may also be true for the forest ant. In a previous study on the regulation of fluid secretion in Malpighian tubules of *F. polyctena* by exo- and endogenous factors (De Decker, 1993), two myotropic peptides were purified based on their myotropic effects on the hindgut of *L. maderae*. These peptides belong to the family of the tachykinins. They were purified from an acidic aqueous ant's head extract and had no effect on the primary urine production in the Malpighian tubules of the ant. In the same extract chromatographic fractions with both diuretic and myotropic effects on ant tubules were found when the extract was fractionated on an analytical C_{18} column (De Decker, 1993). Moreover, a diuretic factor without myotropic activity was purified from the same head extract.

In the same study it was shown that head extracts of the ant also contain **antidiuretic factors**, besides the diuretic factors described above (De Decker, 1993),(De Decker et al., 1994). The presence of antidiuretic factor(s) in the forest ant *F. polyctena* was inferred from the finding that the fluid secretion rate of freshly dissected Malpighian tubules could be very variable (Van Kerkhove et al., 1989). A striking observation was that very often the tubules of animals, two hours after they had been captured from the nest, were completely silent : no secretion could be obtained at all. The Malpighian tubules of these ants were morphologically different, they were white and opaque compared to the brownisch-yellow colour and translucent appearance of the secreting tubules. The next day

tubules of the same batch of animals had resumed fluid secretion. So, apparantly the ants possess a mechanism to reduce the primary urine production by the Malpighian tubules. Since the Malpighian tubules lie free in the abdominal cavity, it is to be expected that the factor(s) responsible for this antidiuretic effect will be found in the ant's hemolymph. So far no endogenous factor with a direct inhibitory effect on fluid secretion has been purified, although antidiuretic effects have been described for crude extracts of *Acheta domesticus* (Spring and Clark, 1990) and *A. aegypti* (Petzel and Conlon, 1991). An inhibitory effect of exogenous peptides on the cell transport mechanisms, in Malpighian tubules of insects, has been described for the cardioacceleratory peptide (CAP_{2b}) on *Rhodnius prolixus* tubules (Quinlan et al., 1997) and for destruxins on *Schistocerca gregaria* tubules (James et al., 1993). CAP_{2b}, originally isolated from the hawkmoth *M. sexta* (Davies et al., 1995), belongs to a group of peptides that are believed to be involved in the regulation of cardiac function, in wing inflation after adult emergence and in thermoregulation during flight. Destruxins are cyclic peptide toxins isolated from the insect pathogenic fungus *Metarhizium anisopliae.* These peptides exerted their effect via an increase in intracellular cGMP (Davies et al., 1995),(Quinlan et al., 1997). In the ant, an antidiuretic effect has been described for high concentrations (10^{-3}-10^{-4}M) of the cell permeable cAMP analogue, chlorophenylthio-cyclic AMP (cpt-cAMP) (De Decker, 1993).
With the present study we intended to further elaborate this antidiuretic effect, with respect to the identification of the antidiuretic factor as well as its mode of action.
A second important aim of this study was to try and isolate endogenous diuretic factors. Also, a start was made in order to find out whether the dual control mechanism, described for the diuretic response of insect Malpighian tubules to myotropic and CRF-related peptides, was present in ant tubules.

II. AIM OF THE PRESENT STUDY

With this study we intended to purify endogenous factors from body extracts of the forest ant, in order to clarify the neuroendocrine control of primary urine formation in ants. The table in the bookmark, gives an overview of the factors known up to now, that play a role in the neuroendocrine regulation of primary urine production in insects. The table compares the results obtained in the present study with the situation found in other insects. The presence of antidiuretic factors in crude extracts from the head or the hemolymph of the ant (De Decker, 1993), lead us to try and purify such a factor, named FopADF (chapter

1). A start was made in elucidating the cell membrane properties affected by this factor (chapter 1).

In search for a second messenger for FopADF, the nitric oxide (NO) - cGMP , the cAMP and the Ca^{2+} pathway were investigated (chapter 2).

An attempt was made to further characterize a diuretic factor (Fop-DP) described by De Decker *et al.* (1993) (De Decker et al., 1993) (chapter 3). It was also assessed whether Ca^{2+} was the second messenger. Furthermore it was investigated whether FopDP had an additive effect on cAMP stimulated tubules (chapter 3).

Larger insects provide more material in the search for neuroendocrine substances in body extracts. Therefore the presence of factors (antidiuretic or diuretic) was investigated in the honeybee, *Apis mellifera*, belonging to the same insect order, i.e. the Hymenoptera. Factors found in the ant were compared to those found in the honeybee (chapter 4).

As there was a possibility that the dual control system (myokinins and CRF-related factors) of the fluid secretion also existed in the ant and as myotropic factors have been characterized previously, it seemed worthwhile to try and find a CRF-related factor in head extracts of the ant (chapter 5). Chromatographic and immunological techniques were used to assess the presence of CRF-related material in an ant's head extract.

Finally head extracts were treated with a purification procedure (chapter 6), with which CRF-related peptides had been isolated in other species. A peptide (Fop-cGP) was isolated using cross-reactivity with the migratory locust. The second messenger pathway for this factor was investigated. Its possible physiological relevance was discussed.

III. OVERVIEW OF THE METHODOLOGY USED

To study the endocrine regulation of the primary urine production in the Malpighian tubules of *F. polyctena*, a number of different experimental approaches were used.

The extraction procedure used is a first important step in the purification of endogenous factors from body extracts of *F. polyctena*. Since we were interested in water-soluble low molecular weight substances, an acidic aqueous extraction solvent was used, i.e. 10-15% trifluoroacetic acid (TFA) in MiliQ (Millipore) water. The membrane lipids and membrane associated components will be insoluble in this solution and the larger proteins will precipitate, due to pH-denaturation. Besides the extraction solvent used, contamination of the crude extract with irrelevant material can be reduced by the sample preparation. If one knows the site of synthesis or the storage site of the material one wants to purify, microdissection of the considered cells or organs, will simplify the ultimate purification. In insects the corpora alata - corpora cardiaca complex, is the major storage and release site of neuroendocriene factors (Orchard, 1983). Microdissection of this organ, combined with an acidic aqueous extraction solvent, a solid phase extraction and a one step high performance liquid chromatography (HPLC) step lead to the succesfull identification of the hypertrehalosaemic peptides in cockroaches (Gäde, 1989). The forest ants are too small for this approach. So instead of using the isolated organ as source tissue, whole heads, thoraces or abdomina were used (Fig. 0.3), following the method described by De Decker *et al.* (1993).

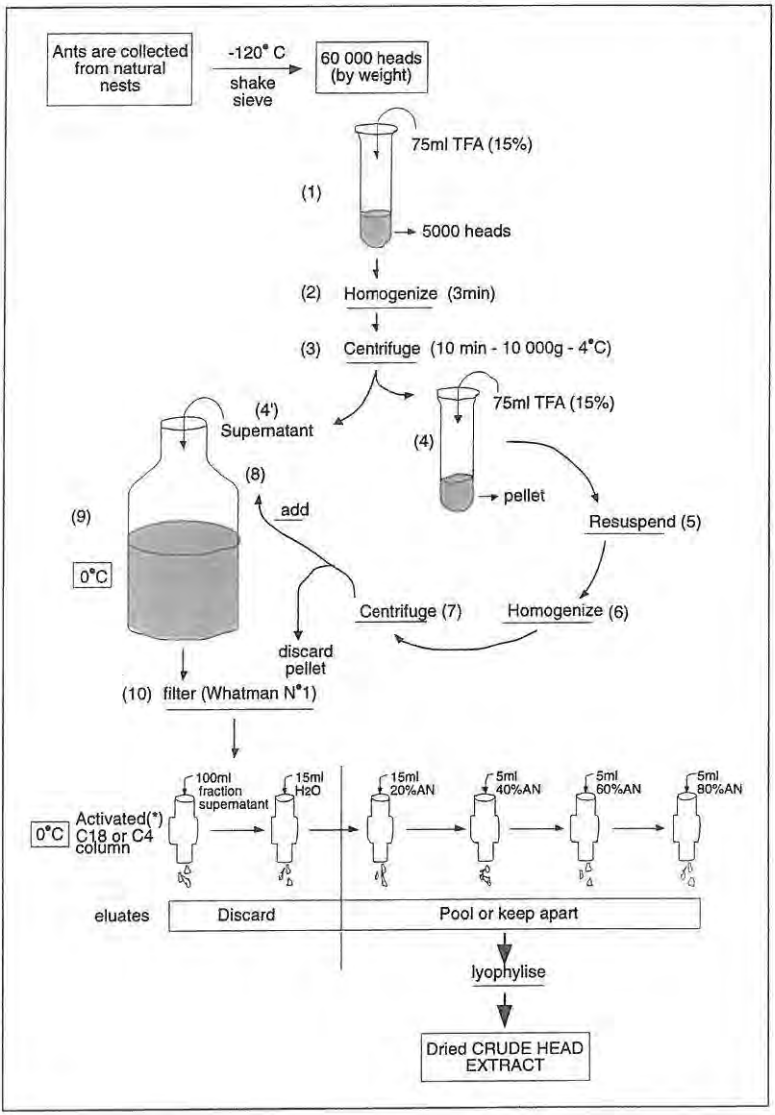

Figure 0.3 Procedure for preparing crude extracts from heads of *Formica*. For more detailed information see "Materials and Methods" of chapter one (p.20).

Further **purification** was obtained with solid-phase extraction on reversed-phase aliphatic C_4 or C_{18} cartridges. Biogenic amines, steroid-like substances and polypeptides are retained on these cartridges. They could subsequently be recovered and fractionated according to their hydrophobicity using a stepwise elution with solvents with an increasing concentration of acetonitrile (CH_3CN). The section "Materials and Methods" in the subsequent chapters includes a detailed description of the used extraction procedures. All of them were based on the procedure represented in figure 0.3, with minor adaptations according to the tissue used.

The prepurified fractions obtained after solid phase extraction were further purified, using **reversed-phase HPLC** on a Waters system consisting of two 510 pumps, a 486 tuneable absorbance detector set at 214 nm and the Millennium™ 2.15 chromatography manager. In reversed-phase chromatography the peptides were retained by the solid phase of the columns. Subsequently a solvent gradient was applied, characterized by an increase in hydrophobicity. As a consequence the material originally bound to the column, eluted according to its solubility characteristics in the mobile phase. The choice of reversed-phase columns with large (>300Å) pore size packing (Holman and Hayes, 1996) was yet another contribution to the success of the chromatographic purification. Without this precaution biological activity was greatly diminished or even lost, due to irreversible peptide adsorption throughout the chromatographic procedures. Purification of the peptides was obtained by changing the column characteristics or by changing the gradients used. The chromatographic fractions obtained were screened for their effects on single isolated Malpighian tubules, by specific and sensitive **bioassays**.

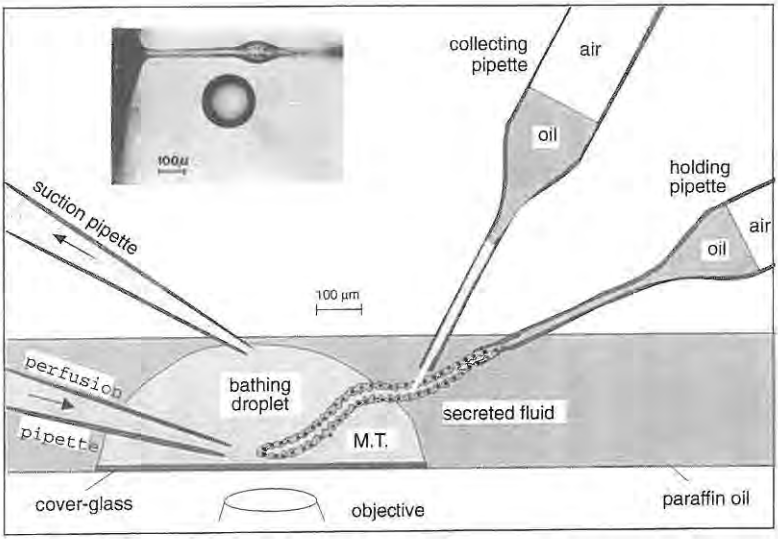

Figure 0.4 Experimental set-up for fluid secretion measurements. Malpighian tubule (M.T.). (Reproduced from Van Kerkhove *et al.*, 1989)

A first important bioassay was **the fluid secretion assay** on single isolated Malpighian tubules. It was based on the Ramsay technique, first described for the Malpighian tubules of the stick insect, *Dixippus morosus* (Ramsay, 1953). Figure 0.4 shows the experimental set-up to study the fluid secretion of *Formica* Malpighian tubules. More detailed information is given in the "Material and Methods" section of the chapters one, two, three and five. The use of an autologous assay to screen for endogenous factors with a direct effect on the primary urine production should result in fractions which have similar effects in *vivo* (Coast et al., 1992). Although used to screen for diuretic activity in chromatographic fractions of large, crude, insect body extracts, it seemed that the fluid secretion assay was not very specific (Spring and Kim, 1995), nearly every fraction tested showed some diuretic or antidiuretic activity. A second disadvantage was that the ant tubule assay is time consuming: only one Malpighian tubule could be tested at a time. Knowing that each experiment takes about two hours, it is obvious that this assay is not very suitable for screening purposes. No other fast, sensitive and reliable bioassay on *Formica* tubules was available however, so the fluid secretion experiments were carried out.

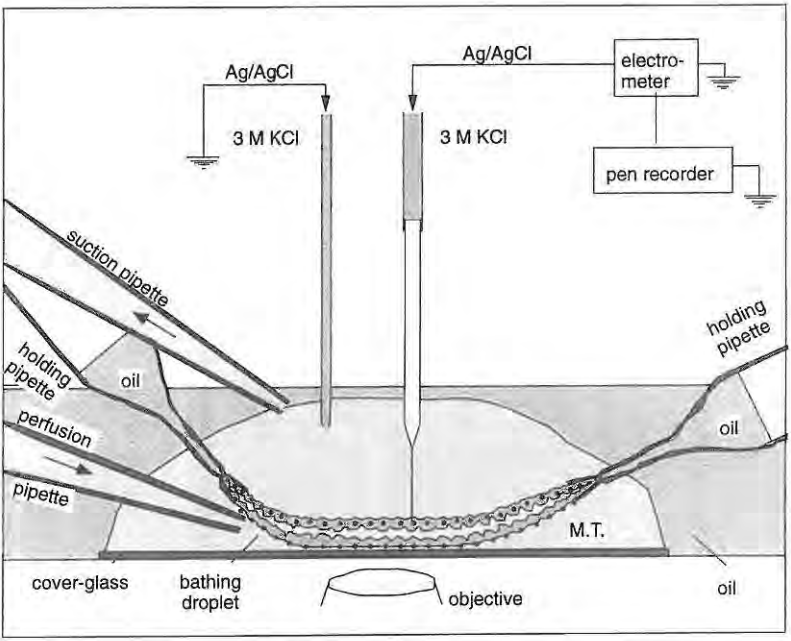

Figure 0.5 Experimental set-up for the measurement of basolateral and transepithelial potential differences. Only one microelectrode is shown. Malpighian tubule (M.T.). (For detailed information, see chaper one p.23). (Reproduced from Van Kerkhove *et al.*, 1989)

To start elucidating the mode of action of different test substances in the Malpighian tubule, the effect on the transepithelial and basolateral membrane **potential differences** was assessed and used as a second bioassay. Potential differences were measured according to the method described by Leyssens *et al.* (1992) (Leyssens et al., 1992). Basolateral (V_{bl}) membrane and transepithelial (V_{te}) potentials were measured simultaneously using conventional microelectrodes. In figure 0.5 only one microelectrode is shown. More detailed information on this assay is given in the "Material and Methods" section of the chapters one and four. It is a very sensitive assay, but has more or less the same disadvantages as the fluid secretion assay, only one experiment can be carried out at the same time. However it should be possible to adapt this assay to the assay described for Malpighian tubules of the yellow-fever mosquito, *A. aegypti* (Petzel et al., 1985). In this

assay the lumen and hemolymph side of the tubules were perfused with symmetrical solutions and the transepithelial potential was measured via a Ag-AgCl electrode in the perfusion pipette. With this assay more than 100 fractions eluting from a single HPLC column could be scanned, using only one or two Malpighian tubules. For future purifications it seems worthwhile to test this approach on *Formica* tubules.

Besides these general procedures, a number of specific procedures, related to the specific problems studied in a chapter, were used. A detailed description of the protein determination, the cAMP determination, the 'enzyme linked immuno sorbent assay' (ELISA), the myotropic assay on *Locust* Malpighian tubules and the secretion experiments on *L. migratoria* Malpighian tubules can be found in the "Materials and Methods" section of chapters two, five and six respectively.

CHAPTER 1

An antidiuretic factor of the forest ant : purification and physiological effects on the Malpighian tubules.

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ABSTRACT

Formica polyctena antidiuretic factor (FopADF) was purified out of a 15% trifluoroacetic acid (TFA) abdomen extract from 150,000 worker ants. After solid phase extraction of the crude extract, 3 different reversed-phase columns were used to purify FopADF. At a concentration of 1.0 ant-equivalents/µl (ant-eq/µl) the factor reversibly inhibited fluid secretion of single isolated Malpighian tubules to 29% ± 5 (n=24) of the control value. Applying FopADF at a concentration of 1.0 ant-eq/µl to the Malpighian tubules reversibly depolarized both the basolateral membrane potential (V_{bl}) from -21mV ± 2 to -3mV ± 1 (n=5) and the apical membrane potential (V_{ap}) from -64mV ± 7 to -19mV ± 5 (n=5). Similar effects were reported previously for the application of dinitrophenol together with barium to the tubules. This suggests that FopADF reduces the active potassium transport by blocking both the apical V-type H+ ATPase and the basolateral potassium channels.

Keywords :

Formica polyctena, High Performance Liquid Chromatography, fluid secretion, electrophysiology.

THE EXCRETORY SYSTEM in insects consists of the Malpighian tubules and the hindgut. The primary urine produced by the Malpighian tubules is voided into the hindgut. The ileum and rectum are responsible for the reabsorption of solutes and water (Gäde et al., 1997),(Pannabecker, 1995).

As in vertebrates, the invertebrate excretory system is under neuroendocrine control by diuretic and antidiuretic hormones (Spring, 1990). Some controversy exists regarding the definition of the terms 'diuretic' and 'antidiuretic' in insects. In this study we define them as stimulatory or inhibitory of the primary urine production by the Malpighian tubule. It is known that diuretic hormones are released in response to feeding (Audsley et al., 1997) or at the time of eclosion from the pupal stage (Nicolson, 1976). A remarkable example is the effect of a blood meal in a hematophagous insect such as *Rhodnius prolixus* : the release of diuretic hormones results in an increase in Malpighian tubule primary urine production with two or three orders of magnitude (Maddrell et al., 1993). Feeding may act as a stimulus for the release of hormones which act on the hindgut to increase fluid reabsorption (Audsley et al., 1994). Of these hormones the neuroparsins are well characterized. These are peptidergic hormones which have a direct stimulatory effect on the reabsorption of KCl by the rectum (Girardie and Fournier, 1993).

Only a few papers report the existence of factors in insect body extracts with an antidiuretic effect on the primary urine production of single isolated Malpighian tubules.

Antidiuresis has been demonstrated in crickets (Spring and Clark, 1990), mosquitos (Petzel and Conlon, 1991) and in ants (De Decker et al., 1994), but the identity of the factors involved was unknown.

The presence of an antidiuretic factor in the forest ant *F. polyctena* was inferred from the finding that the fluid secretion rate of freshly dissected Malpighian tubules could be very variable (Van Kerkhove et al., 1989). A striking observation was that very often the tubules of animals, two hours after they had been captured from the nest, were completely silent : no secretion could be obtained at all. The Malpighian tubules of these ants were morphologically different, they were white and opaque compared to the brownisch-yellow colour and translucent appearance of the secreting tubules. The next day tubules of the same batch of animals had resumed fluid secretion.

This observation led to the hypothesis that stress conditions induced the release of antidiuretic factors into the ant's hemolymph. Two events could trigger this release. A first event could involve the defence mechanism to predators disturbing the nest (Löfqvist, 1976): the ants spray formic acid and empty their poison gland when threatened. The gland's volume is approximately 10 percent of the hemolymph volume (Graedel and

Eisner, 1988). To prevent a dramatic change in body water content and in hemolymph composition, an uptake of water is needed and the production of primary urine must be suppressed. A second problem occurs during winter. The worker ants hybernate at the base of the nest and reactivate the colony during spring (Hölldobler et al, 1990). During winter water preservation is of prime importance for the ants and antidiuretic factors may help to reduce the loss of body fluid. In this paper we present evidence for the existence of an antidiuretic factor in the ant. Based on electrophysiological measurements we propose that its mode of action involves the apical V-type H+ ATPase and the potassium channels in the basolateral membrane.

METHODS

Insects

The ants used in the experiments were collected from their natural nest in the forest of Slederlo (Genk, Belgium). They were reared at 25°C and 65% relative humidity under a 16h:8h light:dark cycle. They were fed a diet of sugar (cubes) and water *ad libidum*.

Saline

The composition of the insect saline was based on the composition of the ant's hemolymph (Van Kerkhove et al., 1989), and contained (in mmol/l) : 27.0 KCl, 8.04 K_3citrate, 1.97 $CaCl_2$, 13.0 $MgCl_2$, 16.8 Na_2fumarate, 14.4 Na_2succinate, 2.80 L-alanine, 10.5 trehalose, 11.7 maltose, 138.8 glucose and 12.1 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES). The saline had an osmolality of 350 mosm/kg and was adjusted to a pH of 7.2 by means of NaOH. The K^+ and Cl^- were somewhat higher than the mean concentrations found in the animal (10-70 mM K^+ and 20-60 mM Cl^-) (Van Kerkhove et al., 1989), but permitted us to start a secretion experiment at an easily measured rate and providing a reliable control value for the tubule under study.

Preparation of a 15% trifluoroacetic acid (TFA) hemolymph extract

Batches of approximately 500 living ants were collected from the natural nest in the forest of Slederlo (Genk, Belgium). Within the two hours following captivity, the Malpighian tubules of 100 ants of each batch were checked under a binocular microscope. Non-secreting tubules could easily be recognised since they were white and opaque compared to

brownish-yellow and translucent for secreting tubules. If more than 65% of the ants had non-secreting tubules, we used the batch to prepare a hemolymph extract.
To collect the hemolymph 50 ants were decapitated and transferred to a small Petri-dish filled with 15 ml of 15% TFA, kept on ice. The abdomina were opened by means of two forceps and the hemolymph collected by gently stirring the abdomen in the solution. The mixture obtained was centrifuged in a Beckmann JA14 rotor at 14,000 rpm for 15 min. The supernatant was collected and filtered in sequence over Whatman N°42 and Whatman N°1 fiter paper prior to prepurification over SepPak Vac 35cc C_{18} Cartridges (Waters) as described below.

Preparation of a 15% TFA abdomen extract
Over a period of three weeks 150,000 ants from the species *F. polyctena* were collected from their natural nest in Slederlo (Genk, Belgium).
They were frozen instantly and kept in liquid nitrogen until further use. The frozen body parts could be separated from one another by sieving. The abdomina were retained with a 1.7 mm sieve, the heads and thoraces with a 1.4 mm sieve. Heads and thoraces were kept in liquid nitrogen until further use.
Batches of 5,000 abdomina (50 g) were transferred into 250 ml centrifugation bottles, crushed by means of a pestle and 120 ml of an icecold 15 % TFA solution was added.
This mixture was shaken vigourously and left on ice for 10 min prior to centrifugation.
The bottles were centrifuged in a Beckmann JA14 rotor at 14,000 rpm for 15 min.
The supernatant was collected and the pellet washed twice with 120 ml of icecold 15% TFA.
The collected supernatant was filtered in sequence over Whatman N°42 and Whatman N°1 filter paper prior to prepurification over SepPak Vac 35cc C_{18} Cartridges (Waters). The cartridges had been wetted previously with acetonitrile (CH_3CN) and were equilibrated in milliQ (Millipore) water. The cartridges were eluted stepwise with 90 ml of 20%, 40%, 60% and 80% CH_3CN in 0.1% TFA. The fractions were lyophilized in a Heto-Vac system and stored in the freezer at -70 °C for further use.

Chromatographic purification of FopADF
F. polyctena antidiuretic factor (FopADF) was purified with reversed-phase high performance liquid chromatography (RP HPLC) on a Waters equipment consisting of two 510 pumps and a 486 tunable absorbance detector set at 214 nm. Data acquisition and analysis was performed using the Millennium™ 2.15 chromatography manager software.

Samples, containing 10,000 to 15,000 ant-equivalents (ant-eq) of the lyophilized 60% CH_3CN fraction, were resuspended in 40 ml of 5% CH_3CN, 0.1% TFA. This solution was loaded on a preparative Waters Delta-Pak C_{18} (15µm, 100Å, 25x100 mm) column, using one of the HLPC solvent delivery pumps. A linear gradient of 5 - 80% CH_3CN in constant 0.1% aqueous TFA over 160 min was used for the chromatographic separation (flow rate of 6 ml/min). Fractions were collected every two minutes with a Waters 5.302 Fraction Collector. Samples of these fractions, containing 2000 ant-eq were dried in polypropylene Eppendorf tubes by centrifugal evaporation. The dried fractions were redissolved in insect saline and used in the fluid secretion bioassay on single isolated Malpighian tubules as described below.

The fraction of the preparative run with the most pronounced antidiuretic effect in the bioassay was concentrated by means of lyophilization. The lyophilized material was redissolved in 5% CH_3CN in 0.1% aqueous TFA and loaded on an analytical Waters Delta-Pak HPI C_{18} (5µm, 300Å, 3.9x150 mm) column as described above. The column was eluted with a linear gradient of 5 - 80% CH_3CN in constant 0.1% aqueous TFA over 50 min, with a flow rate of 1ml/min. Peak fractions were collected manually and prepared for assay as described above.

Only one fraction from the analytical C_{18} column showed significant activity and was finally purified on a Pharmacia Sephasil Protein C4 (5µm, 300Å, 4.6x100 mm) column. A linear gradient of 20-80% CH_3CN in constant 0.05% aqueous TFA over 60 min. A flow rate of 0.5 ml/min was applied. Peak fractions were collected manually and prepared for assay as described above.

Fluid secretion assay for a single isolated Malpighian tubule

The midgut with the Malpighian tubules still attached to it was dissected out of an ant in fresh insect saline. One Malpighian tubule was cut free and transferred into a bathing droplet of approximately 50 µl in a Petri-dish, placed on the stand of an inverted microscope (Zeiss). The bathing droplet was covered with paraffin oil and the open end of the tubule was pulled out into the oil by means of a holding pipette. The bathing droplet was perfused with insect saline at a rate of 200 µl/min. To collect the secreted fluid the Malpighian tubule was punctured with a silanized collection pipette (1,1,1,3,3,3-hexamethyldisilizane). The secreted fluid of the first 10 minute period was discarded. Subsequently droplets were collected every 10 minutes. The collected fluid was blown out of the collection pipette under oil and formed a perfect sphere on the tip of the collection

pipette. The diameter was measured with an eyepiece micrometer and secretion rate calculated.

Each experiment consisted of three periods of three times 10 minutes. The first period was a control period of which the third collection was taken as a reference. Subsequently the perfusion was switched off and the bathing droplet replaced with 50 µl of test solution. The test solution contained redissolved chromatographic fractions. During the test period the bath solution was not renewed. Stopping the bath perfusion on its own did not affect the fluid secretion rate as seen in a set of 35 control experiments (results not shown). Fluid secretion rate was expressed as a percentage of the reference value (third collection of the control period). During the last period of 30 minutes the continuous perfusion with insect saline was switched on again and the test solution was washed out of the bathing droplet.

Measurement of membrane potentials

A Malpighian tubule was dissected out of an ant in fresh insect saline and transferred to a bathing droplet (approximately 50 µl) covered with paraffin oil. The Malpighian tubule was immobilized at the bottom of the bath by means of two holding pipettes. This immobilization was essential to prevent tubule movement due to the continuous perfusion of the bath with insect saline.

Intracellular (V_{bl}) and transepithelial (V_{te}) potentials were measured using two conventional microelectrodes (borosilicate filament glass, Hilgenberg, FRG; OD 1.5 mm, ID 1 mm; tip diameter < 0.5 µm) filled with 3 M KCl. These high resistance (20 - 40 MΩ) measuring microelectrodes were connected to a dual probe electrometer (WPI Model M750) via a Ag/AgCl wire. A low resistance (1 MΩ) reference microelectrode filled with 3M KCl and connected to the ground via a Ag/AgCl wire closed the electrical circuit. The first microelectrode was pushed through the cell layer into the lumen and measured V_{te}. Subsequently a cell was impaled with the second microelectrode to measure V_{bl}. The impalement was accepted if a sudden negative deflection occurred that remained stable for at least a few minutes.

The results were recorded on a dual-pen recorder (Philips Model PMB 8252). They were accepted only if the microelectrode potential differed not more than ± 4 mV from zero after withdrawal of the microelectrode at the end of the experiment. The apical membrane potential (V_{ap}) was calculated as the difference between V_{bl} and V_{te} ($V_{ap} = V_{bl} - V_{te}$).

Each experiment consisted of three periods lasting 10 to 15 minutes : a control, a test and a washout period. After the control period the bath perfusion was switched off, the bathing droplet removed and the test solution added. Stopping the bath perfusion on its own did

not affect the membrane potentials as seen in a set of ten control experiments (results not shown). Finally the test solution was washed out of the bathing droplet by resuming the continuous perfusion with insect saline.

Statistics

Results are given as mean values ± standard error (S.E.M.), with the number of tubules in parentheses. Statistical significance was calculated using the paired Student's *t*-test, unless indicated otherwise. A difference was considered significant if $p \leq 0.05$.

RESULTS

Biological effect of the 15% TFA hemolymph extract

In animals with non-secreting tubules it is to be expected that antidiuretic factors have been released into the hemolymph (De Decker et al., 1994). Therefore a small 15% TFA extract was made from the hemolymph of a batch of 200 animals with silent tubules. The prepurified fractions were pooled and the resulting crude extract was tested for its effects both on the fluid secretion and on the membrane potentials.

In the fluid secretion assay single isolated active Malpighian tubules were challenged with a test solution containing 1.0 ant-equivalents/µl (ant-eq/µl) of the crude hemolymph extract.

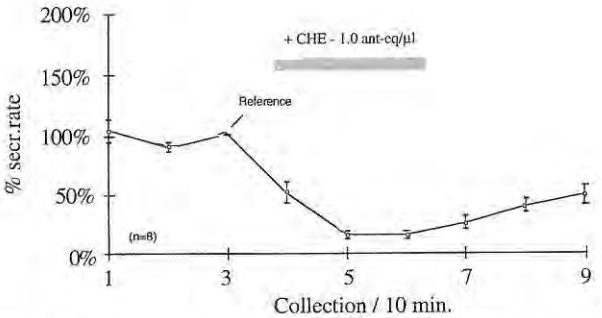

Fig. 1.1 Effect of 1.0 ant-eq/µl of crude hemolymph extract (CHE) on the fluid secretion rate of isolated Malpighian tubules. Presence of the extract is indicated by the bar. Mean values ± S.E.M., (n=number of tubules tested).

The extract reduced the fluid secretion of active tubules with 76% ± 4 (n = 8, $p<0.001$). Upon wash out the fluid secretion only partially recovered to a value of 47% ± 8 (n=8) (Fig. 1.1). This slow recovery may be due to the presence of toxic components in the crude extract. Tested at the same concentration the extract also depolarized V_{te} with 14.8 mV ± 2.6 (n=8) (Fig. 1.2). The effect did not significantly recover. The extract had no significant effect on the basolateral membrane potential, so the drop in V_{te} was due to a depolarisation of the apical membrane potential.

Prepurification of the abdominal extract

Collection of hemolymph fluid from the abdomen is time consuming. To obtain a large amount of material a 15% TFA extract was made of whole abdomina of 150,000 ants that had been frozen and sieved. Prepurification of the crude extract over the C_{18} cartridges resulted in four separate CH_3CN fractions (20, 40, 60, and 80%) characterized by a stepwise increase in hydrophobicity of the eluted material. The different prepurified fractions were tested in the fluid secretion assay (Table 1).

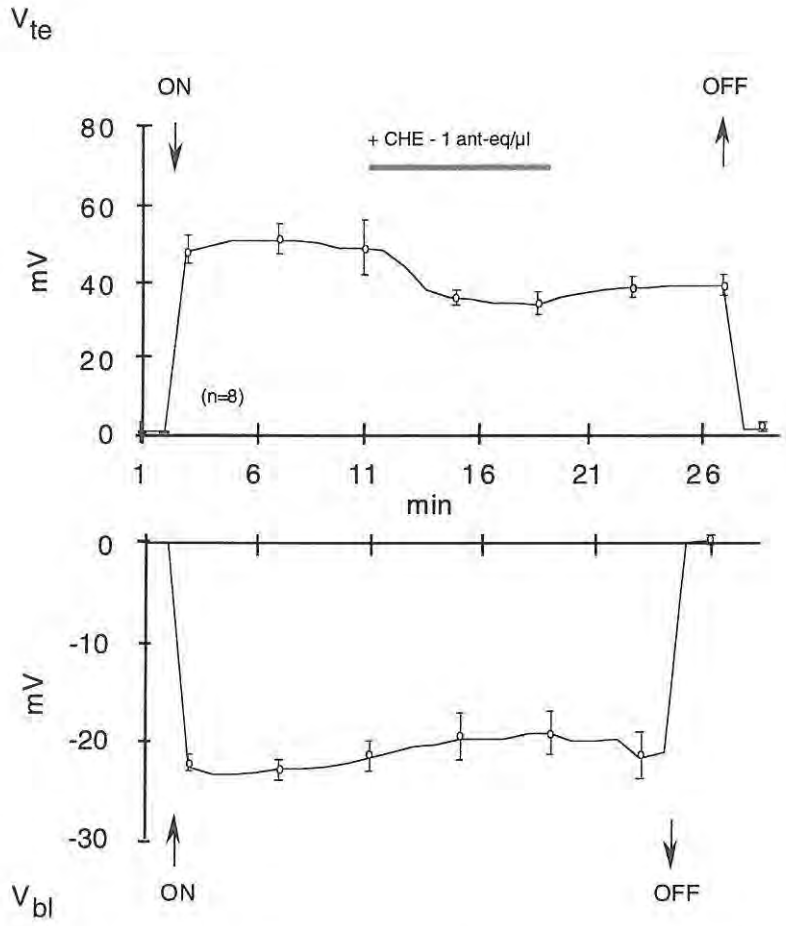

Fig. 1.2 Running mean for the simultaneous effect of crude hemolymph extract (CHE), tested at a concentration of 1 ant-eq/µl, on the transepithelial (Vte) and the basolateral (Vbl) membrane potential. Presence of the extract is indicated by the bar. Arrows indicate impalement (ON) or withdrawal (OFF) of the microelectrodes. Mean values ± S.E.M., (n=number of tubules tested).

The 60% SepPak fraction seemed to have an irreversible effect on the fluid secretion rate which became evident only during washout. During the test period no significant effect on the primary urine production was found, but during the washout period the fluid secretion was significantly reduced. The 20% fraction had a stimulatory effect which persisted throughout the washout period. The 40% fraction provoked a dual response, the stimulatory response seen during the test period was lost and followed by a severe reduction of fluid secretion during the washout period. The 80% fraction had no significant effect on the fluid secretion rate. These results are similar to those obtained with a 10% TFA head / thorax extract of the ant (Laenen et al., 1998) and suggest that the abdominal extract contained both diuretic and antidiuretic factors. The 60% fraction seemed to have the highest content of antidiuretic factor(s), it was consequently used for the chromatographic HPLC purification.

Table 1. *Effect of 0.1 ant-eq/µl of the different SepPak fractions on the fluid secretion rate of single isolated Malpighian tubules of the ant.*

	% secretion rate / control			
SepPak Fraction	Control	Test	Recovery	n
20% CH_3CN	104 ± 5	220 ± 21 *	227 ± 16 *	6
40% CH_3CN	99 ± 3	133 ± 13 *	59 ± 15 *	9
60% CH_3CN	100 ± 3	93 ± 10	43 ± 11 *	10
80% CH_3CN	110 ± 9	101 ± 16	82 ± 16	3

The third collection of secreted fluid during the control period was taken as 100%. * Significantly different from the controls (paired Student's *t*-test, p 0.05). Mean values ± S.E.M, n = number of tubules tested.

Chromatographic fractionation of the 60% SepPak fraction

Running the 60% prepurified fraction on the preparative Delta-Pak C_{18} column yielded different fractions that were tested for their effect on the fluid secretion rate in single isolated Malpighian tubules (Fig. 1.3). Only one of them, fraction 7 (Fr.7) significantly inhibited the primary urine production. This fraction had a retention time of 156 min. It eluted at 62% CH_3CN and, when tested at a concentration of 1.0 ant-eq/µl, it irreversibly reduced the fluid secretion rate to a value of 25% ± 5 (n=4, $p<0.01$ paired Student's *t*-test).

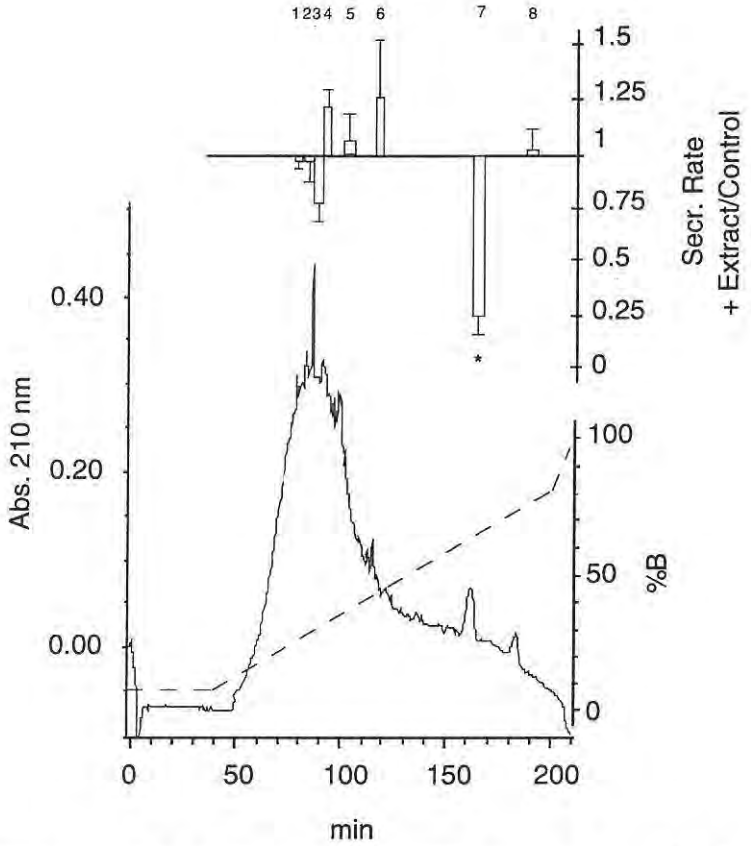

Fig. 1.3 U.V. absorbance profile of 10,000 ant-eq of the 60% SepPak fraction separated on the Waters PrePak DPC18 column (300Å,25x100mm). Conditions are described in Materials and Methods. The dashed line represents the concentration of solvent B. The upper part shows the effect of the fractions, tested at a concentration of 1.0 ant-eq/µl, on fluid secretion by isolated Malpighian tubules (mean values ± S.E.M., n = 4 tubules). The effect is expressed as the ratio of fluid secretion rate in the presence of the fraction over the reference value during the control period. * Significantly different from 1 ($p<0.01$, paired Student's *t*-test).

To process the total crude extract 9 preparative runs had to be performed. Fr.7 was pooled, concentrated by centrifugal evaporation and temporarely stored in 50% CH_3CN at -20°C prior to the lyophilization needed to prepare the material for the next purification step.

Chromatographic separation of the Fr.7 pool on the analytical Waters Delta-Pak HPI C_{18} column revealed only one major U.V.-absorbing peak. This peak fraction had an inhibitory effect on the primary urine production, but in contrast to Fr.7 the effect was reversible. Applied at a concentration of 1.0 ant-eq/µl it reduced the fluid secretion rate to 29% ± 5 (n=24, $p<0.01$ paired Student's *t*-test) (recovery to 83% ± 5 after washout) (Fig. 1.4). Based on this biological effect we named this factor *Formica polyctena* Antidiuretic Factor (FopADF).

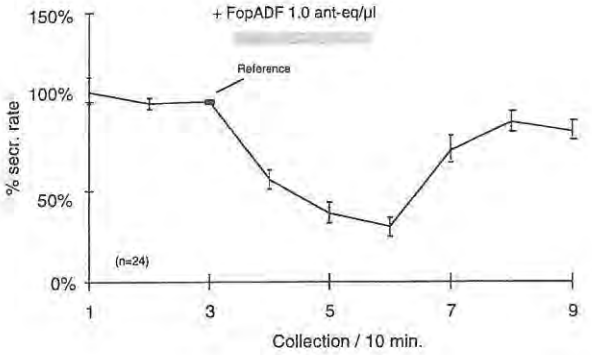

Fig. 1.4 Effect of FopADF, tested at a concentration of 1 ant-eq/µl on fluid secretion by isolated Malpighian tubules of the forest ant Formica polyctena. Presence of the factor is indicated by the bar. Values represent mean ± S.E.M., (n=number of tubules tested).

FopADF was finally fractionated on a Sephasil Protein C_4 column (Fig. 1.5). Remarkable was the fact that after this last step we needed to apply it at a much higher concentration to pick up the antidiuretic effect in the bioassay. A concentration of 20 ant-eq/µl was needed to reduce the primary urine production to a value of 45% ± 15 (n=4, $p<0.05$ paired Student's *t*-test) (recovery to 93% ± 5 after washout).

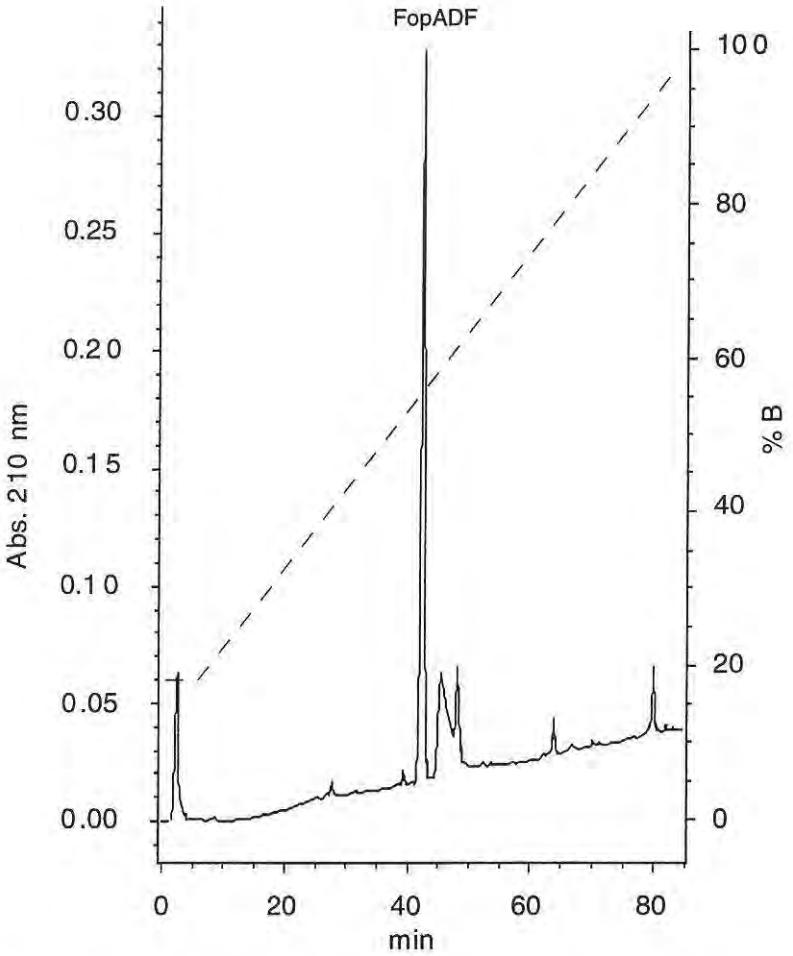

Fig. 1.5 U.V. absorbance profile of 40,000 ant-eq of FopADF separated on the Sephasil Protein C4 (5µm, 300Å, 4.6x100mm) column. Conditions are described in Materials and methods. The dashed line represents the concentration of solvent B.

Membrane potential profile of silent and actively secreting tubules

The membrane potential profile of non-secreting tubules (Fig. 1.6A) differed markedly from the potential profile of secreting tubules as shown in Fig. 1.6B. As was shown previously (Leyssens et al., 1992) the potential profile of secreting tubules is similar to that reported for other insects. It shows a large, cell negative, apical membrane potential (V_{ap}) and a smaller basolateral membrane potential (V_{bl}). Consequently the transepithelial membrane potential (V_{te}) is positive (lumen with respect to the bath).

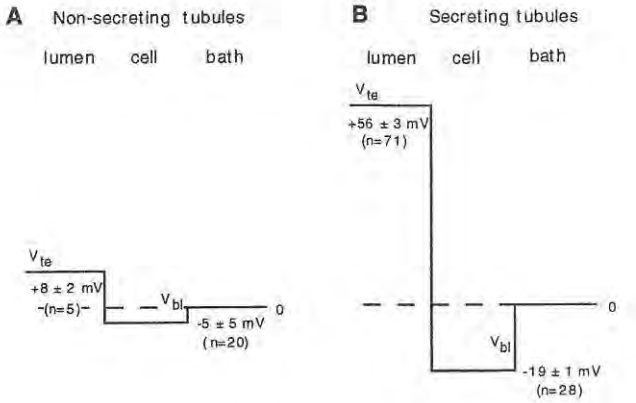

Fig. 1.6: potential profile of non-secreting (A) and secreting Malpighian tubules (B). Mean values ± S.E.M., (n=number of tubules tested) (Reproduced from De Decker, 1993).

In non-secreting tubules both membrane potentials are much smaller than those of secreting tubules. It was therefore of interest to investigate the effect of FopADF on V_{ap} and V_{bl} of secreting tubules.

Effect of FopADF on the membrane potentials

Freshly extracted FopADF, purified on the analytical C_{18} column, was used to study its effect on the membrane potentials of single isolated Malpighian tubules. FopADF was applied at a concentration of 1 ant-eq/µl to secreting tubules (Fig. 1.7). A reversible depolarisation of both the basolateral membrane potential (from -21mV ± 2 to -3mV ± 1, n=5) and of the apical membrane potential (from -65mV ± 5 to -20mV ± 5, n=5) was observed. The potential profile (Fig. 1.7, insert) after treatment with FopADF, was comparable to the profile of non-secreting tubules (Fig. 1.6A) : the difference was not significant (p=0.76, unpaired Student's *t*-test). So FopADF may be one of the key factors explaining the ant's ability to block its primary urine production.

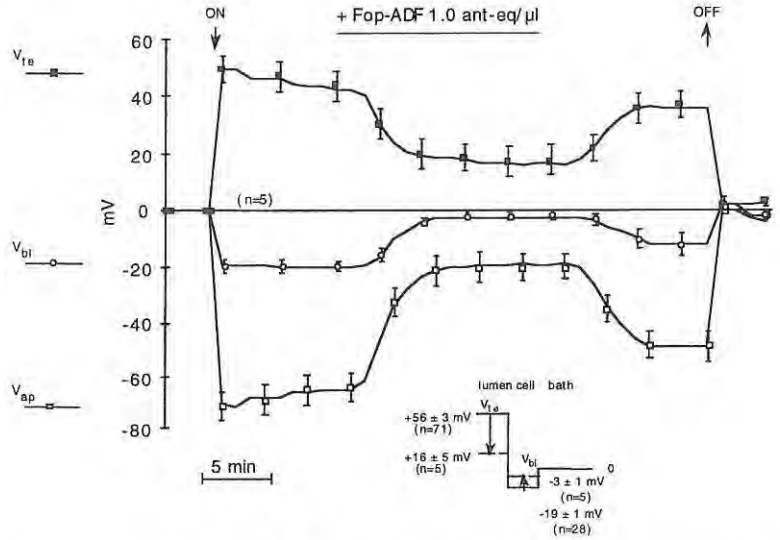

Fig. 1.7: Running mean for the effect of FopADF, tested at a concentration of 1 ant-eq/µl on the transepithelial (Vte) and the basolateral (Vbl) membrane potential measured simultaneously. The apical membrane potential (Vap) was calculated as Vap = Vbl - Vte. Presence of the factor is indicated by the bar. Arrows indicate impalement (ON) or withdrawal (OFF) of the microelectrodes . Mean values ± S.E.M., (n=number of tubules tested). Insert : potential profile of the tubules in control condition (full line) or in the presence of FopADF (dotted line). Mean values ± S.E.M.

DISCUSSION

Purification of FopADF

An antidiuretic factor was purified with a three step HPLC protocol. FopADF had a reversible, inhibitory effect on the primary urine production by the Malpighian tubules.

So far no endogenous factor with a direct inhibitory effect on fluid secretion has been purified, although antidiuretic effects have been described for crude extracts of *Acheta domesticus* (Spring and Clark, 1990), *Aedes aegypti* (Petzel and Conlon, 1991) and *F. polyctena* (De Decker et al., 1994). Inhibitory effects, for exogenous peptides on the transport mechanisms in the Malpighian tubules of insects, have been described for the cardioaccelatory peptide (CAP_{2b}) on *R. prolixus* tubules (Quinlan et al., 1997) and for destruxins on *Schistocerca gregaria* tubules (James et al., 1993).

CAP_{2b}, originally isolated from the hawkmoth *Manduca sexta* (Davies et al., 1995), belongs to a group of peptides that are believed to be involved in the regulation of cardiac function, in wing inflation after adult emergence and in thermoregulation during flight. So far only two CAPs have been sequenced: CAP_{2a} (Cheung et al., 1992) a cyclic nonapeptide and CAP_{2b} (Huesmann et al., 1995), an octapeptide blocked at both the amino and carboxyl ends. CAP_{2a} and CAP_{2b} have no sequence homology. Destruxins are cyclic peptide toxins isolated from the insect pathogenic fungus *Metarhizium anisopliae*. Their isolation was based on the paralysing effect on silkworms due to a calcium-dependent depolarisation of the muscle cells (Kodaira, 1961).

When we take a closer look at the molecular characteristics of these peptides, some common features become apparent. Both CAP_{2b} and destruxins are small peptidergic molecules that are well protected from peptidase activity : CAP_{2b}, since both the amino and carboxyl terminus are blocked, and destruxins, since they are cyclic peptides. Although they are small molecules, they elute from the Waters SepPak C_{18} cartridges in the 60% CH_3CN fraction (Cheung et al., 1992; Huesmann et al., 1995; Kodaira et al., 1961) indicating that these substances are quite hydrophobic. The importance of this characteristic in relation to the physiological function still has to be elucidated.

The elution behaviour of FopADF is comparable to the elution pattern of the peptides described above. Consequently if the above assumptions are correct, then FopADF might be a small amidated peptide blocked at the amino terminus with pyroglutamate or blocked by cyclisation. This shielding of the N-terminus may explain why we could not obtain sequence information using standard Edman degradation techniques (results not shown).

Other methods may be needed (e.g. Mass Spectrometry (MS) - MS analysis) in order to obtain an amino acid sequence for FopADF.

Mode of action of FopADF : a model

In previous studies a model for KCl transport in *F. polyctena* Malpighian tubules has been proposed (Van Kerkhove, 1994). In this model potassium is transported against a steep electrochemical gradient, against both a concentration and an electrical gradient. The active translocation of potassium from hemolymph to lumen is parallelled by passive transport of Cl^- and water across the epithelium.

The transcellular transport of potassium consists of K^+ uptake at the basolateral membrane and a K^+/H^+ exchange via an antiporter at the apical membrane. A V-type H^+ ATPase in the apical membrane creates an electrochemical H^+-gradient directed from lumen to cell (Zhang et al., 1994). This electrochemical gradient can be used by the antiporter to extrude K^+ from the cell into the lumen, in exchange for protons. The basolateral uptake of potassium occurs primarily through channels, at least in the presence of a high K^+ concentration. At lower hemolymph potassium concentrations electroneutral K^+/Cl^- and $Na^+/K^+/2Cl^-$ cotransporters become important (Leyssens et al., 1994).

According to this model FopADF could affect either K^+ uptake, K^+ extrusion or both. The prime mover, i.e. the V-type H^+ ATPase, is also a likely candidate for regulation of salt and fluid secretion in the Malpighian tubules of the ants. The finding that FopADF reduced V_{ap} indicated that the proton pump may indeed be a target for the antidiuretic factor. Similar effects (depolarization of V_{ap}) were found for different drugs with an inhibitory effect on the apical H^+-pump. Two typical V-type H^+ ATPase inhibitors, N-ethylmaleimide (NEM) and bafilomycin-A1 (Baf-A1) depolarised the apical membrane potential and reduced the fluid secretion rate (Weltens et al., 1992) in single isolated Malpighian tubules. None of these substances had an effect on the basolateral membrane potential. Another way to block the pump and cause a depolarization of V_{ap}, was by applying dinitrophenol (DNP). DNP had indirect inhibitory effects on the apical H^+-pump, through the depletion of ATP (Gennis, 1989) or as an uncoupler of the apical H^+-gradient (Dijkstra et al., 1994). Like NEM and Baf-A1, DNP did not affect V_{bl} (Leyssens et al., 1993). The effects of an inhibition of the apical pump on the basolateral membrane potential were small or absent, indicating that blocking the V-type H^+ ATPase on its own, is not enough to mimick the behaviour of FopADF.

The same conclusion stands for the effect of barium, a blocker of the basolateral K^+ channels (Leyssens et al., 1993). Barium caused an inhibition of fluid secretion, but a pronounced hyperpolarization of V_{bl} and V_{ap}. Blocking of the K^+ channels in the basolateral membrane allowed the electrogenic V-type H^+ ATPase to build up a higher electrical potential difference across the apical membrane (Weltens et al., 1992). So a larger part of the electromotive force of the pump was spent in increasing V_{ap} and as a consequence less energy was left to build the proton concentration gradient necessary to drive K^+ extrusion by the electroneutral apical K^+/ H^+ exchanger (Leyssens et al., 1993). The result was a reduced fluid secretion. The reduced K^+ conductance in the presence of barium, also slowed down basolateral K^+ entry, notwithstanding a pronounced increase in the cell inward electrochemical gradient for K^+ (Weltens et al., 1992).

Only when DNP and Ba^{2+} were applied at the same time, a depolarization of both V_{ap} an V_{bl} could be observed (Figure 9 in reference (Leyssens et al., 1993)). The resulting potential profile was similar ($p=0.66$, unpaired Student's *t*-test with Welch's correction) to the potential profile obtained after treatment of secreting tubules with FopADF (Fig. 1.7, insert). These results suggest that FopADF slowed down K^+ secretion, possibly by reducing both the K^+ conductance in the basolateral membrane and the electrogenic proton pump activity in the apical membrane.

A possible physiological role of antidiuretic factors

The physiological role of antidiuretic factors with a direct inhibitory effect on the Malpighian tubules of insects still has to be elucidated. In crickets the release of an antidiuretic hormone from corpora cardiaca was triggered by the hydration state of the animal (Spring and Clark, 1990). The release could be triggered indirectly by the decline in hemolymph volume and reduced distention of the abdomen, sensed by abdominal stretch receptors as reported for the antidiuretic effect of CAP_{2b} in *R. prolixus* (Quinlan et al., 1997). In ants FopADF seemed to be released after emptying of the poison gland. The volume of this gland represents approximately 10 percent of the abdominal volume (Graedel and Eisner, 1988). An observation showing that the ants experienced water loss was that the ants drank a lot of water during the first 12 hours upon captivity from their natural nest (unpublished observations). So a change in abdominal volume could explain the release of FopADF in *F. polyctena*. The antidiuretic factor(s) may be important to prevent a further reduction in body fluid. Release of regulatory factors on a change in abdominal volume was also seen in hematophagous insects which needed to reduce their

hemolymph volume after a blood meal (Maddrell et al., 1993),(Wheelock et al., 1988). Further research is necessary to confirm this hypothesis in the ants

Conclusion

This is the first report on the purification of an endogenous antidiuretic factor in an insect. FopADF, was purified from an abdominal extract of *F. polyctena*. FopADF had a reversible inhibitory effect on the fluid secretion rate of *F. polyctena* Malpighian tubules and it depolarised both the apical and the basolateral membrane potential of the tubules in a reversible way. The factor may be responsible for the inactivation of the primary urine production in ants, in a stress situation.

Based on the electrophysiological findings we propose that FopADF inhibits the active potassium secretion by blocking both the apical V-type H^+ ATPase and the basolateral potassium channels. Since an inhibitory effect on the primary urine formation of secreting tubules was observed for a hemolymph extract collected from stressed animals, the physiological role of the antidiuretic factor may be prevention of further fluid loss, after emptying of the poison gland in stress conditions.

ACKNOWLEDGEMENTS

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EFFECT OF FopADF ON THE MALPIGHIAN TUBULES OF THE MEALWORM *TENEBRIO MOLITOR*

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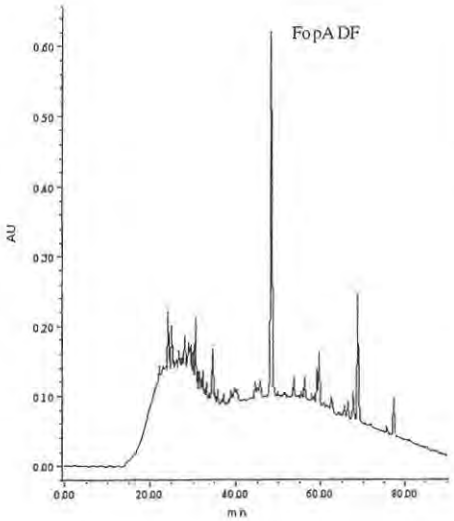
Chromatographic purification of FopADF

A 10% TFA abdomen extract was made from a batch of 5,000 ant's, following the procedure described in the Materials and Methods section of chapter 1. Two thousand five hundred ant-eq of the 65% CH_3CN fraction were purified on an analytical Waters Delta-Pak HPI C18 (5µm, 300Å, 3.9x150 mm) column using the conditions described in the Materials and Methods section of chapter 1. The peak fraction containing FopADF was collected manually and 2 µl of a 0.5% BSA solution was added. Lyophilisation was done in polypropylene Eppendorf tubes by centrifugal evaporation.



Resulting chromatogram for 2500 anteqivalents of the 65% CH_3CN fraction on the Waters Delta-Pak HPI C18 (5µm, 300Å, 3.9x150 mm) column.

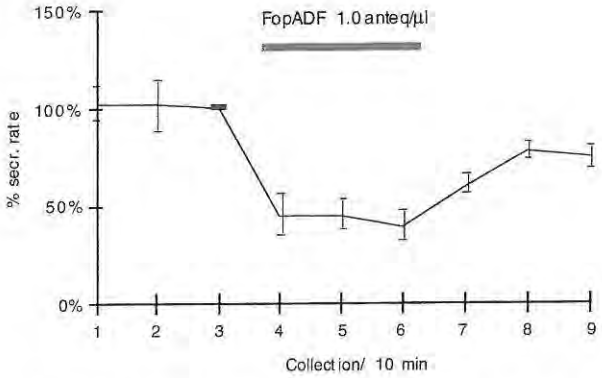
Mobile Phase :
A : 0.1 % TFA
B : 100 % CH_3CN, 0.1 % TFA

Gradient :
0 - 10 min. : 5 % B
10 - 60 min. : 5 - 80 % B
60 - 100 min. : 80 - 100 % B

Flow Rate : 1 ml/ min.

Checking the biological effect of FopADF

One sample was tested in the fluid secretion assay on single isolated Malpighian tubules of the ant to verify its activity. Tested at a concentration of 1 ant-eq/µl it reduced the fluid secretion rate to 43% ± 8 (n=5, $p<0.01$ paired Student t-test) compared to the internal control.

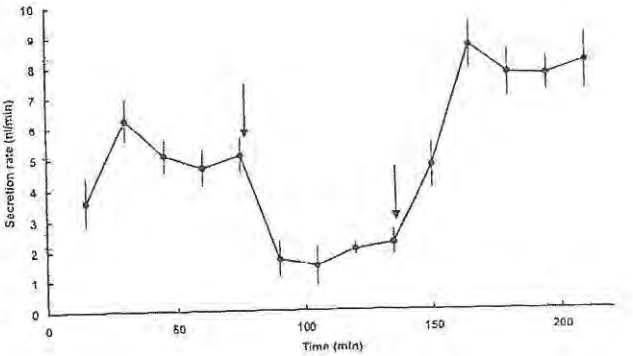


Effect of FopADF, tested at a concentration of 1.0 ant-eq/µl on fluid secretion by isolated Malpighian tubules of the forest ant *Formica polyctena*. Presence of the factor is indicated by the bar. Values represent mean ± S.E.M., 5 tubules were tested.

Cross reactivity of FopADF on Malpighian tubules of T. molitor.

The rest of the material was shipped to the laboratory of Dr. S. Nicolson (Cape Town, South-Africa) and tested at a concentration of 4 ant-eq/µl on single isolated Malpighian tubules of the mealworm. The fluid secretion rate was reduced to 44% ±9 (n=4) compared to the final collection of the control period. At the end of the test period, FopADF was washed out and replaced by 1mM cAMP. As expected the fluid secretion rate was stimulated to 165% ± 18 (n=4).

So FopADF inhibited the fluid secretion to the same extent as in the ant and the effect was not toxic: fluid secretion resumed after washout and could be stimulated with cAMP.



Effect of FopADF, tested at a concentration of 4.0 ant-eq/µl on fluid secretion by isolated Malpighian tubules of the mealworm *Tenebrio molitor*. Adding of the factor is indicated by the first arrow. The second arrow indicates the removal of FopADF and the adding of 1 mM cAMP. Values represent mean ± S.E.M., 4 tubules were tested.

CHAPTER 2

Investigation of signal transduction pathways in the Malpighian tubules of the forest ant: role in or interaction with the antidiuretic factor, FopADF

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Running head: Signal transduction pathways

ABSTRACT

In search for a signal transduction pathway for the *Formica polyctena* antidiuretic factor (FopADF) in the Malpighian tubule of the ant, three second messenger pathways (cAMP, calcium, NO-cGMP) known to occur in insect Malpighian tubules, have been investigated.

An involvement of cAMP could be excluded, firstly because the antidiuretic effect of FopADF was not suppressed in the presence of SQ22536, an adenylate cyclase inhibitor and secondly since FopADF had no effect on the intracellular cAMP concentration as assessed in a I^{125}-cAMP competition assay.

The NO-pathway was tested at two different points of interference. Neither L-arginine, the natural substrate for NO-synthetase, nor SNAP, a NO-releasing agent, affected the fluid secretion rate in single isolated Malpighian tubules. Apparently the NO-pathway could not be triggered with an effect on fluid secretion in ant tubules and as such is an unlikely candidate for the signal transduction pathway of FopADF.

Finally the effects of changes in intracellular calcium were analyzed. Increasing intracellular calcium stimulated fluid secretion and the stimulatory effect of A23187, a calcium ionophore, could be blocked through the presence of FopADF in the test medium. The results suggest that FopADF affects cellular mechanisms beyond those stimulated by a rise in cytoplasmic calcium or cAMP.

INTRODUCTION

Primary urine formation by the insect Malpighian tubule is controlled by endogenous factors, mostly small peptides (for reviews see references ((Beyenbach, 1995),(Coast, 1996),(Gäde et al., 1997)). Best characterized are the peptides with a direct stimulatory effect on the fluid secretion rate of single isolated Malpighian tubules, the diuretic hormones. They can be grouped in two families, one family has sequence and structure homology with the human corticotropin releasing factor (CRF) / sauvagine / urotensin-I family of vertebrate peptides and is consequently named the CRF-related family of diuretic peptides (Coast et al., 1994). The second family, the myokinins are named according to their myotropic effect on the hindgut of the cockroach, *Leucophea maderae* (Schoofs et al., 1993). Each family operates via a specific second messenger. The CRF-related peptides were shown to bind to G-protein coupled receptors, activating adenylate cyclase. This resulted in an increase of intracellular levels of cAMP (Reagan, 1995). The myokinins seem to act through intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) since their actions are indistinguishable from those of calcium mobilizing drugs: indirect manipulation of the $[Ca^{2+}]_i$ concentration with ionophores such as A23187, or calcium mobilizing agents such as thapsigargin caused a stimulation of fluid secretion (Coast, 1995),(O'Donnell et al., 1996).

Another group of low molecular weight peptides, the cardioacceleratory peptides or CAPs use a different signal transduction pathway. These peptides have been isolated from the tobacco hornworm *Manduca sexta* (Cheung et al., 1992) and are believed to stimulate the heart immediately after adult emergence, facilitating wing inflation. They are also active during flight in order to achieve sufficiently rapid hemolymph circulation between abdomen and thorax for thermoregulation (Tublitz et al., 1991). One of them, CAP_{2b}, has an effect on insect Malpighian tubule fluid secretion. Recent work by Dow *et al.* (1994) showed that CAP_{2b} elevated the cGMP levels in *Drosophila melanogaster* tubules. The increase of this nucleotide affected the apical V-type H^+-ATPase and resulted in an increased primary urine production (Dow et al., 1994). In tubules of the blood-feeding insect *Rhodnius prolixus* CAP_{2b} elevated the cGMP levels also, but in this case the primary urine production was reduced (Quinlan et al., 1997). The difference between *Drosophila* and *Rhodnius* tubules may lie in the receptor for CAP_{2b} and the intracellular pathway that is activated. In *Drosophila* tubules the diuretic effect of CAP_{2b} was mimicked by the NO-releasing agent sodium nitroprusside (SNP) suggesting

that NO sensitive guanylate cyclase is involved in the diuretic response of CAP_{2b} (Quinlan et al., 1997). Additional proof was presented by Davies *et al.* (1995) in fluid secretion experiments with methylene blue, a specific inhibitor of NO sensitive guanylate cyclase (Audsley et al., 1997). Since it blocked the effects of CAP_{2b} on the primary urine production, it was concluded that the NO-cGMP pathway is the signal transduction pathway used by the cardioactive peptide in *Drosophila*.

In *Rhodnius* tubules SNP had no effect on the fluid secretion rate and exogenously applied 8-Br-cGMP reduced fluid secretion in *Rhodnius* tubules (Quinlan et al., 1997). This indicated that the increase in intracellular cGMP did not occur via a NO sensitive guanylate cyclase, but possibly via a membrane-bound guanylate cyclase. A conclusion prompted by the fact that cGMP synthesis is controlled by two classes of guanylate cyclase (Schulz et al., 1991): a group of soluble enzymes stimulated by the highly diffusible NO (Severina, 1998), and a family of membrane-bound enzymes which are stimulated by peptide agonists such as atrial natriuretic factor (ANF) (Chinkers et al., 1989).

Another exogenous peptide, the cyclic peptide toxin, destruxin A, inhibited fluid secretion in Malpighian tubules of the desert locust (James et al., 1993). Destruxin A was isolated from the insect pathogenic fungus *Metarhizium anisopliae* (Kodaira, 1961). Its isolation was based on the paralysing effect on silkworms due to a calcium-dependent depolarisation of the muscle cells. The toxin inhibited the primary urine production in locust Malpighian tubules, in a cAMP and calcium independent way. The stimulation of fluid secretion by cAMP in the presence of the cAMP phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX) was abolished by destruxin A. The inhibition of fluid secretion by destruxin A could not be prevented by the presence of a calcium channel blocker nor by the absence of external calcium (James et al., 1993). Involvement of cGMP, by analogy with *Rhodnius*, is not very likely, as cGMP-analogues have a diuretic effect on locust Malpighian tubules (Morgan and Mordue, 1984).

From the results reported above it could be concluded that there is not necessarily a clear correlation between antidiuresis and a particular signal transduction pathway in insect Malpighian tubules. In the present study all three second messenger pathways (cAMP, calcium, NO-cGMP) have been investigated in the Malpighian tubules of *F. polyctena* and their role in the action of FopADF has been assessed.

MATERIALS AND METHODS

Insects

The ants used in the experiments were collected from their natural nest in the forest of Slederlo (Genk, Belgium). They were reared at 25°C and 65% relative humidity under a 16h:8h light:dark cycle. They were fed a diet of sugar and water.

Experimental solutions

The composition of the insect saline was based on the composition of the ants hemolymph (Van Kerkhove et al., 1995) and contained (in mmol/l) : 27.0 KCl, 8.04 K_3citrate, 1.97 $CaCl_2$, 13.0 $MgCl_2$, 16.8 Na_2fumarate, 14.4 Na_2succinate, 2.80 L-alanine, 10.5 trehalose, 11.7 maltose, 138.8 glucose and 12.1 Hepes. The saline had an osmolality of 350 mosm/kg and was adjusted to a pH of 7.2 by means of NaOH.
Chlorophenylthio-cyclic AMP (cpt-cAMP), A23187, IBMX, forskolin and L-arginine were purchased from Sigma. Thapsigargin and SQ22536 were purchased from Calbiochem. S-nitroso-N-acetylpenicillamine (SNAP) was purchased from Biomol. FopADF was freshly extracted and purified on the Delta-Pak HPI C_{18} column, as described previously (Laenen et al., 1998b). All substances (except IBMX, forskolin, SQ22536 and thapsigargin) were dissolved in the insect saline, concentrations used are specified in the Results section. IBMX, forskolin, SQ22536 and thapsigargin were prepared as a stock solution in DMSO (dimethyl sulfoxide : Across Chimica). Prior to use the stock solution was diluted with insect saline and the final concentration of DMSO was 1 °/$_{oo}$ or less. Final concentrations of test substances used are specified in the Results section. The effect of 1 °/$_{oo}$ DMSO was tested in control experiments. No significant effect was seen on the secretion rate (Table 2) of single isolated Malpighian tubules.

Fluid secretion assay for a single isolated Malpighian tubule

To measure the fluid secretion of a single isolated Malpighian tubule of the ant, we used the method described by De Decker *et al.* 1994 (De Decker et al., 1994). A small adaptation had to be made in order to work with two test solutions. When two test solutions were used, the experiment consisted of four periods of three times 10 minutes. The first period was a control period, the third collection of which was taken as a reference. The two following periods were test periods. At the start of the test periods the bathing droplet was sucked away and replaced with 50 µl of test solution. Only small amounts of test solution were available, which made it impossible to sustain a continuous

bath perfusion. Stopping the bath perfusion on its own did not significantly affect the secretion rate, but was somewhat smaller at the end of these long experiments, as seen in a set of ten control experiments (see Results section). The effect of a test substance was expressed as the percentage with which fluid secretion changed with respect to the reference. In the final period, i.e. the washout period, the test solution was washed out and replaced with control saline. During the control and washout period the bath was perfused with insect saline at a rate of 200 µl/min.

Determination of intracellular cAMP concentrations

The Malpighian tubules of three ants (i.e. circa 45 tubules in all) were pooled in a petri-dish filled with fresh insect saline. The tubules were transferred to an eppendorf tube together with 25 µl of saline and kept on ice. When all the tubules were collected, the reaction vials were synchronised by a preincubation of 15 minutes at room temperature. In order to inhibit phosphodiesterase activity, IBMX was added to each of the tubes (final concentration of 10^{-4}M). After a second incubation period of 15 min, 15 µl of insect saline with or without test sample was added to the tubes which were incubated for another 30 minutes. Addition of 900 µl of ice-cold methanol and transfer to ice for 15 minutes stopped the reaction and precipitated the tissue proteins. The cAMP formed was liberated by sonication (3 times 30 seconds). The samples were centrifuged (13.000 rpm for 15 min), the supernatant was lyophilized and its cAMP content was determined by means of a I^{125} cAMP scintillation proximity assay kit, according to the manufacturer's instructions (Amersham International, RPA538). The protein content of the pellets was determined by means of a BCA-protein determination kit (Pierce, N°23223) with bovine serum albumin (BSA) as a standard.

Statistics

All values are reported as means ± standard errors of the mean (S.E.M.). Statistical comparisons were performed using the paired Student's *t*-test, unless indicated otherwise. Probalities less than 0.05 were considered significant.

RESULTS

Effect of cpt-cAMP on the primary urine production

To investigate the influence of an increased intracellular cAMP concentration on the ant Malpighian tubules, the cAMP analogue cpt-cAMP was used. Cpt-cAMP is a cAMP

derivative that permeates the cell membrane much faster than cAMP and that is not easily broken down. It was tested at two concentrations 10^{-3}M and 10^{-5}M, and had a dual effect on the primary urine production in ant tubules. At a concentration of 10^{-5}M cpt-cAMP stimulated the fluid secretion, with a slow return to the control values during washout (Table 1).

Table 1. *Effect of different test substances on the fluid secretion rate of single isolated Malpighian tubules of the ant.*

	% secretion rate			
Test Substance	Control Period	Test Period	Washout Period	n
insect saline	122 ± 5	105 ± 11	110 ± 11	35
cpt-cAMP 10^{-3}M A	140 ± 21	102 ± 6	240 ± 34 *, †	4
cpt-cAMP 10^{-3}M B	112 ± 1	65 ± 5 *	135 ± 9 *, †	6
cpt-cAMP 10^{-3}M C	147	218	284	2
	126	175	246	
cpt-cAMP 10^{-5}M	107 ± 4	142 ± 9 *	100 ± 5 †	8
L-arginine 10^{-4}M	115 ± 14	96 ± 8	84 ± 8 *	6
A23187 10^{-4}M	118 ± 4	218 ± 31 *	96 ± 19 †	9

The third collection of secreted fluid during the control period was taken as 100%.
* Significantly different from the control period (paired Student's *t*-test, $p \leq 0.05$).
† Significantly different from the test period (paired Student's *t*-test, $p \leq 0.05$).
Mean values ± S.E.M, n = number of tubules tested.

However at a concentration of 10^{-3}M the ant tubules responded in three different ways. In 4 out of 12 cases cpt-cAMP had no effect during the test period (cpt-cAMP A in Table 1), in 6 out of 12 cases it even reduced the primary urine production (cpt-cAMP B in Table 1) and for the two remaining cases it increased the fluid secretion (cpt-cAMP C in Table 1), analogous to the response obtained with 10^{-5}M cpt-cAMP (Table 1). In all 12 cases however, fluid secretion increased when cpt-cAMP was washed out of the bathing droplet.

Investigation of the adenylate cyclase system in ant Malpighian tubules

The presence of the adenylate cyclase system in the ant Malpighian tubules was tested with the adenylate cyclase inhibitor, SQ22536, and the adenylate cyclase activator forskolin. The results show that the adenylate cyclase sytem is functional in the Malpighian tubules of *Formica*. In previous experiments De Decker (1993), demonstrated that 10^{-5}M forskolin increased the intracellular cAMP concentration in the Malpighian tubules of the ant. This increase in cAMP is accompanied by an increase in fluid secretion (Fig. 2.1). The same concentration of SQ22536 (10^{-5}M) had no effect on its own but blocked the diuretic effect of forskolin (Fig. 2.1). No significant difference ($p = 0.84$, unpaired Student's *t*-test) was found between the experiments with SQ22536 and those with both SQ22536 and forskolin in the test solution.

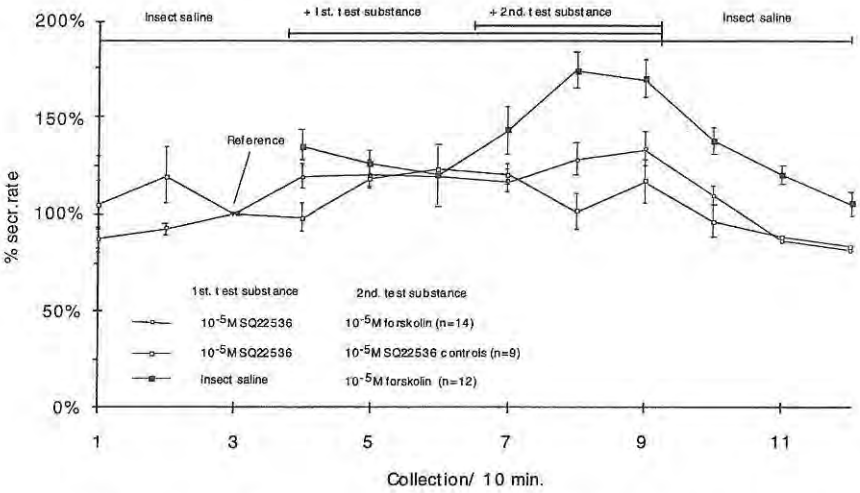

Figure 2.1. Effect of different test substances on the fluid secretion rate of single isolated Malpighian tubules of the forest ant *Formica polyctena*. The presence of the test substances in the bath is indicated by the bars. Mean values ± S.E.M., (n=number of tubules tested).

Does FopADF exert its antidiuretic effect via an increase in the intracellular cAMP concentration ?

In a first set of experiments FopADF was applied first, reducing the fluid secretion. Subsequently cpt-cAMP was added in the presence of FopADF (Fig. 2.2). Since for these experiments the bath perfusion was switched off during 1 hour, a first set of 10 control experiments was performed. A slow decrease of the basal fluid secretion rate with time could be observed (Fig. 2.2). At the end of the second test period it was reduced by 26% ± 5 compared to the internal reference ($p<0.01$, n=10). As a consequence all results obtained for long incubation periods were compared to these control experiments using an unpaired Student's *t*-test.

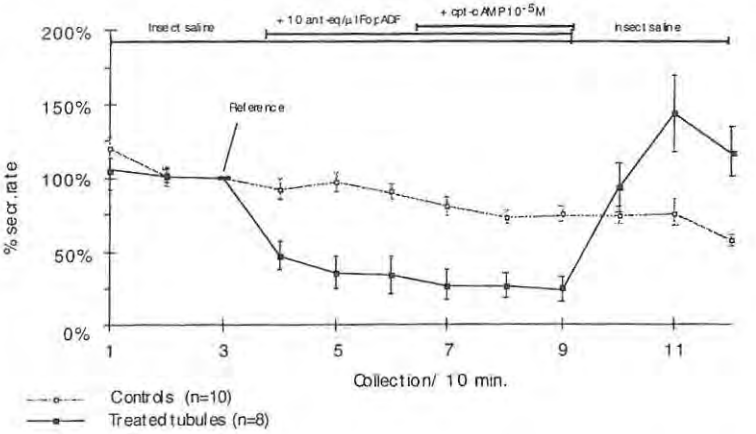

Figure 2.2. Effect of 10^{-5}M cpt-cAMP, in the presence of 10 ant-eq / µl FopADF, on the fluid secretion rate of single isolated Malpighian tubules of the forest ant *Formica polyctena*. The dashed line represents the slow decrease of basal fluid secretion rate as observed in a set of 10 control experiments. The presence of the test substances in the bath, is indicated by the bars. Mean values ± S.E.M., (n=number of tubules tested).

Taking the time effect into account, it was concluded that cpt-cAMP could not overcome the antidiuretic effect of FopADF (Fig. 2.2). Instead of recovering, the fluid secretion was further reduced. The presence of the slowly metabolized cAMP analogue cpt-cAMP became apparent during the washout period. Removal of the antidiuretic factor resulted in an overshoot instead of the expected recovery to the control values (Fig. 2.2).

In a subsequent set of experiments FopADF was added to the Malpighian tubules in the presence of SQ22536. It was found that FopADF does not need the adenylate cyclase sytem to exert its antidiuretic effect: the effect persisted in the presence of the adenylate cyclase inhibitor (Fig. 2.3). So it seems that FopADF is not using cAMP as a second messenger.

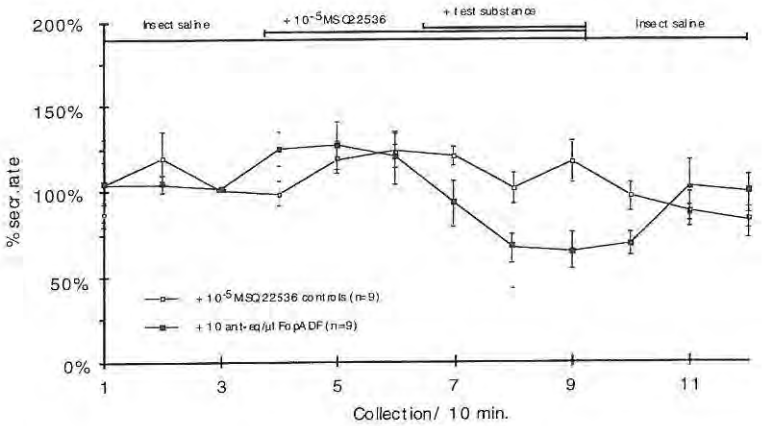

Figure 2.3. Effect of FopADF 10 ant-eq/µl, in the presence of 10^{-5}M SQ22536, on the fluid secretion rate of single isolated Malpighian tubules of the forest ant *Formica polyctena*. The presence of the test substances in the bath, is indicated by the bars. Mean values ± S.E.M., (n=number of tubules tested).

This was confirmed via a competitive cAMP binding assay. The responsiveness of the tubules to 0.1 ant-eq/µl of the 40% CH_3CN fraction, known to contain *F. polyctena* cAMP generating peptide (Laenen et al., 1998a), was taken as a positive control.

Table 2 *cAMP production in Malpighian tubules of the ant in response to different test substances*

Test substance	fmol cAMP / 3 ant-eq	n	pmol cAMP / µg protein
Control : IBMX 10^{-4}M	33 ± 3	20	3.35 ± 0.52
FopADF 0.1 ant-eq/µl	32 ± 2	36	3.25 ± 0.51
40% Fr. 0.1 ant-eq/µl	93 ± 6 *	8	9.45 ± 1.21 *

Mean values ± S.E.M, n = number of tubules tested. * Significantly different from the IBMX control (paired Student's *t*-test, $p \leq 0.05$).

The results (Table 2) show that FopADF had no effect on the cAMP levels when compared to the effect of 10^{-4}M IBMX alone. The 40% CH_3CN fraction on the other hand tripled the intracellular cAMP level in the Malpighian tubules of the ant. Protein determinations were performed in order to express the cAMP results as pmol cAMP / µg protein. The protein content of the Malpighian tubules pooled from three ants equaled 9.84 µg ± 0.63 (n=40) and the average cAMP concentration for the IBMX controls was calculated to be 3.35 pmol/µg protein ± 0.52.

Has the NO-cGMP system a functional role in the regulation of fluid secretion of Malpighian tubules of Formica *?*

In a first set of experiments the natural substrate for NO-synthetase, L-arginine, was used. Based on the reported values in the literature (Dow et al., 1994) it was tested at a concentration of 10^{-4}M and had no effect on the ant tubules (Table 1). In a second series of experiments a NO releasing agent was used. SNAP was tested at three different concentrations. At the highest concentration (10^{-3}M) SNAP had a toxic effect. The secretion rate fell during the test period and did not recover during the washout period. For the other concentrations 10^{-4}M and 10^{-6}M, no effect on the primary urine production was observed (Fig. 2.4). Even an incubation for two hours with 10^{-4}M SNAP had no effect (Fig. 2.5).

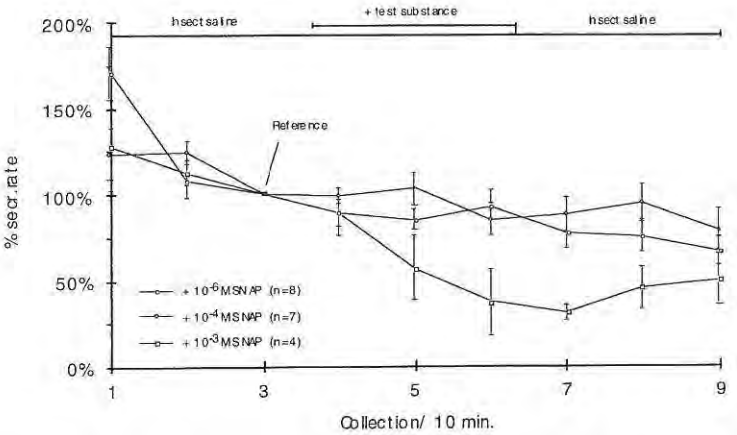

Figure 2.4. Effect of different S-nitroso-N-acetylpenicillamine (SNAP) concentrations on the fluid secretion rate of single isolated Malpighian tubules of the forest ant *Formica polyctena*. The presence of SNAP in the bath, is indicated by the bar. Mean values ± S.E.M., (n=number of tubules tested).

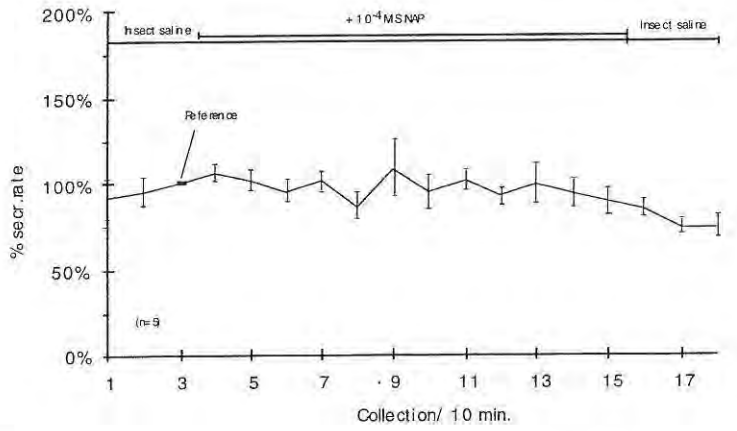

Figure 2.5. Effect of 10-4M S-nitroso-N-acetylpenicillamine (SNAP) on the fluid secretion rate of single isolated Malpighian tubules of the forest ant *Formica polyctena*. The presence of SNAP in the bath, is indicated by the bar. Mean values ± S.E.M., (n=number of tubules tested).

Effects of the calcium ionophore A23187 on the Malpighian tubules of the forest ant

The involvement of calcium was investigated with the calcium ionophore A23187. This component doubled the fluid secretion rate when tested at a concentration of 10^{-4}M in the presence of 1.97 mM Ca^{2+} (Table 1) (see also Chapter 3, p.).

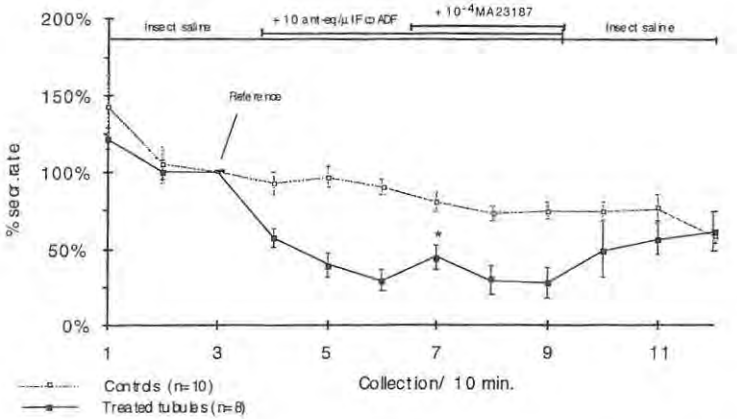

Figure 2.6. Effect of 10^{-4}M A23187, in the presence of 10 ant-eq / µl FopADF, on the fluid secretion rate of single isolated Malpighian tubules of the forest ant *Formica polyctena*. The dashed line represents the slow decrease of basal fluid secretion rate as observed in a set of 10 control experiments. The presence of the test substances in the bath, is indicated by the bars. * Significantly different from the previous collection value. Mean values ± S.E.M., (n=number of tubules tested).

The present series of experiments was designed to find out whether an interaction between FopADF and calcium existed. In these experiments A23187 was added to Malpighian tubules inhibited by 10 ant-eq/µl of FopADF. An increase of intracellular calcium could not restore the fluid secretion in these tubules (Fig. 2.6). Only a small transient increase was observed during the first collection period of the second test period. It changed from 29% ± 7 to 44% ± 8 (n=8, $p \leq 0.05$). The fluid secretion rate then dropped to the previous low level. This might indicate that FopADF blocks the transport mechanisms, stimulated by an increase in intracellular calcium.

DISCUSSION

The dual effect of cpt-cAMP

We closely investigated the cAMP signal transduction pathway. First of all, De Decker *et al.* (1994) found that a hemolymph extract that inhibited fluid secretion also increased the cAMP content of the ant's Malpighian tubules. This might indicate that the antidiuretic factor(s) present in this extract, used cAMP as second messenger. Secondly, the results with cAMP-analogues suggested a dual response of the Malpighian tubules to an increase in intracellular cAMP. It seemed that low intracellular cAMP concentrations stimulated the primary urine production and that high concentrations reduced it. The effect was not toxic as in all cases the primary urine production was stimulated upon washout of 10^{-3}M cpt-cAMP. Apparently upon washout the intracellular concentration of the slowly metabolized cAMP-analogue was lowered to a concentration where it had a diuretic effect. A similar response was obtained for the combined effect of FopADF and cpt-cAMP, after washout of both factors.

A dual response of cAMP had also been observed in endocytotic vesicles obtained from rabbit renal cortex (Gurich and Dubose, 1989). The effect of 8-Br-cAMP on the H+-ATPase was concentration dependent. At a low concentration (5 µM) the proton pump, localised in the membrane of the endosomes, was activated which resulted in a decrease of intravesicular pH. At high concentrations (250 µM) the proton pump was inhibited (Gurich and Dubose, 1989). This difference in response was due to a difference in affinity for cAMP of the subunits of the proton pump. At low concentrations cAMP bound to the 87 kDa subunit, at high concentrations the proton pump was addressed via the 25kDa subunit (Houslay, 1995). A similar reasoning could explain the results obtained for the ant Malpighian tubules.

In the ant tubules the transcellular transport of potassium consists of K+ uptake through potassium channels at the basolateral membrane and of a K+/H+ antiporter at the apical membrane (Weltens et al., 1992). A V-type H+ ATPase, located in the apical membrane, creates an electrochemical H+-gradient directed from lumen to cell (Zhang et al., 1994). This electrochemical gradient is used by the antiporter to extrude K+ from cell to lumen, in exchange for protons. Stimulation of the apical proton pump via a low cAMP concentration would lead to an increase in fluid secretion rate. An inhibition via a high cAMP concentration would cause a reduction.

FopADF and cAMP as signal transduction pathway

From the experiments where FopADF was added to the Malpighian tubules in the presence of SQ22536, it is clear that FopADF does not need the adenylate cyclase system in order to exert its antidiuretic effect. That FopADF is not using cAMP as a second messenger was confirmed in a competitive cAMP binding assay. FopADF had no effect on the average cAMP content of *Formica* tubules.

As a consequence, the cAMP generating activity of the hemolymph extract, reported by De Decker *et al.* (1993), was not due to FopADF present in this extract. So it must have been due to other factor(s) present.

Investigation of the NO-cGMP pathway

The NO-cGMP pathway can be regulated by two distinct NO-generating systems, i.e. two distinct Nitric Oxide Synthetases (NOS) (Busse et al., 1995). Both systems stimulate a soluble guanylate cyclase in the cytosol.

In the first system the NO-cGMP pathway is triggered by a constitutive NOS (cNOS), a membrane associated enzyme responsible for a basal NO-production in the cell.

In the second system NO-activation of the soluble guanylate cyclase is achieved via inducible NOS (iNOS). In contrast to cNOS, iNOS is only present in the cytosol after induction of the iNOS synthesis by growth factors or cAMP. This activation of the iNOS-cGMP pathway has a time lag of 4 - 6 hours and involves activation of tyrosine kinases (Marczin et al., 1993), mRNA transcription and initiation of protein synthesis (Nathan, 1992). We did not try to induce the synthesis of iNOS. iNOS is not expected to be present in the cell.

To try and demonstrate the presence of cNOS, the natural substrate of NOS L-arginine was added. This had no effect on fluid secretion in the ant Malpighian tubules. This result suggested that cNOS, if present, played no role in the regulation of the fluid secretion. This is somewhat surprising since the cNOS-cGMP pathway is an important signal transduction pathway in insects. The cNOS-cGMP pathway has been demonstrated in the signal transduction pathway for the cardioaccelatory peptide CAP_{2b} in *Drosophila* Malpighian tubules (Audsley et al., 1997) and for the eclosion hormone (EH) in the silkworm abdominal ganglia (Shibanaka et al., 1994). It is also involved in the olfactory system of insects and was found in the chemosensory neuropiles of the antennal lobes of *Apis mellifera* and *D. melongaster* (Marczin et al., 1993).

Other substances that may induce the formation of cGMP by the soluble guanylate cyclase are the S-nitrosothiols such as SNAP, S-nitrosocysteine and S-nitroso-2-

mercaptoethylamine or the NO-releasing agents such as sodium nitroprusside (SNP). All of these substances were shown to function as active intermediates in the vasodilator action of organic nitrates and nitrites in bovine coronary artery (Ignarro et al., 1981). Their activity was explained by the fact that in the natural activation of guanylate cyclase, NO primarily reacts with a variety of thiols to form unstable S-nitrosothiols, which are potent activators of guanylate cyclase. As a consequence the formation of NO via a NOS is shunted when S-nitrosothiols are used.

Thus if a NO sensitive soluble guanylate cyclase was present in *Formica* tubules, SNAP should stimulate it as was the case for SNP in *Drosophila* Malpighian tubules (Dow et al., 1994). Besides the toxic effect of the highest test concentration, SNAP had no influence on the fluid secretion in the ant's Malpighian tubules. In *Drosophila* (Dow et al., 1994) a certain delay was observed before the occurrence of an effect of SNP. Therefore long incubation experiments were performed. But even in these experiments no effect on the ant primary urine production could be observed. It could consequently be concluded that, if the NO-cGMP pathway is present in the ant's Malpighian tubule, it does not seem to play a role in the regulation of fluid secretion.

Calcium and antidiuresis in the Malpighian tubules of the ant

In earlier experiments a diuretic peptide was purified from a head/thorax extract of the forest ant (Laenen et al., 1998b). This peptide stimulated the fluid secretion, using calcium as second messenger. This diuresis occured in a cAMP independent way as shown by the additive effect of cpt-cAMP to the stimulation with FopDP, and vice versa (Laenen et al., 1998b). It was proposed that FopDP might affect a Cl^--conductance located in the shunt or the apical membrane, in analogy with the effects of the leucokinins on the Malpighian tubules of the mosquito *Aedes aegypti*. (Wang et al., 1996).

Having an antidiuretic factor which blocked the active secretion processes in the ant tubule it should be possible to expose the possible effects of calcium on the shunt Cl^--conductance in the presence of FopADF. When the calcium ionophore was added to tubules blocked by FopADF, a transient stimulation of the fluid secretion could be observed. Apparently an increase in intracellular calcium still influences transport mechanisms which are only partially blocked by the presence of the antidiuretic factor. If calcium affects an intercellular Cl^--conductance, a prime factor to consider are the changes in the electrochemical gradient for Cl^- in the presence of FopADF. The combined effect of FopADF and the calcium ionophore, on the membrane potentials

should be measured. If an increase in intracellular calcium affects the shunt conductance, an additional depolarisation of the transepithelial potential should be observed.
A second question to adress, is how an increase in intracellular calcium eventually leads to an increase in shunt conductance in Malpighian tubules. Studie of vertebrate tight junctions provide the first ideas for this association. In vertebrate tight junctions are associated with integral and peripheral membrane proteins that interact with the cell cytoskeleton, in particular a perijunctional ring of actin and myosin. Contractions of this perijunctional ring are thought to affect tight junction structure and permeability (Madara, 1989). The dynamic regulation of this ring via intracellular signalling pathways is presently considered to be the clearest mechanism for the regulation of tight junctional permeability (Anderson and Van Itallie, 1995).

Conclusion

The present results suggest that neither cAMP nor calcium participate directly in the reversible inhibitory effect of FopADF on the primary urine production of Malpighian tubules of the forest ant *F. polyctena*: cAMP, since FopADF did not affect its intracellular concentration compared to the IBMX treated controls and calcium, since an increase in intracellular calcium still had a transient stimulatory effect on the fluid secretion in the presence of FopADF. Similar conclusions were reached for the inhibition of fluid secretion in *Schistocerca gregaria* (James et al., 1993) by cyclic peptide toxins, destruxins and for the inhibition of ecdysteroid secretion in *M. sexta* prothoracic glands (Sloman and Reynolds, 1993) by destruxins. In both cases it was suggested that the toxins act at a level beyond the control of either calcium or cAMP.
In an effort to investigate the NO-cGMP pathway it was concluded that the latter could not be triggered in a detectable way in the ant tubules and that it is an unlikely candidate for the signal transduction pathway of FopADF.

ACKNOWLEDGEMENTS

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CHAPTER 3

Partial identification of a peptide that stimulates the primary urine production of single isolated Malpighian tubules of the forest ant, *Formica polyctena*.

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ABSTRACT

A peptide was purified from a 10% trifluoroacetic acid (TFA) head/thorax extract of 300,000 ants with high performance liquid chromatography (HPLC). Fluid secretion assay of single isolated Malpighian tubules was used as a bioassay. The purity of *F. polyctena* diuretic peptide (Fop-DP) after a two step HPLC protocol was confirmed by means of mass spectrometry and revealed a molecular mass of 7,514 daltons. Due to lack of material, no enzymatic digestion could be performed and the sequence of only the first 25 amino acids could be determined : VPKYENCVSEVLPAGDRQRCVKVTC. A computer search of sequence data banks did not reveal any significant similarity between Fop-DP and other known insect diuretic peptides.

Fop-DP had no effect on the basolateral membrane potential and depolarised the apical membrane potential of the Malpighian tubule cells. This effect as well as the stimulatory effect on the primary urine formation in the Malpighian tubule of the ant, could be mimicked with A23187, a calcium ionophore, and by thapsigargin, an inhibitor of the endoplasmic reticulum calcium ATPase. Fop-DP did not stimulate the cAMP content. The results suggest that Fop-DP uses an increase of intracellular calcium as cellular transduction mechanism.

Keywords :
Hymenoptera, diuretic factor, high performance liquid chromatography, cysteine rich peptide, electrophysiology

INTRODUCTION

In insects the excretory system consists of the Malpighian tubules and the hindgut (ileum and rectum) (Phillips, 1981). The Malpighian tubules produce the primary urine by secretion, driven by the active transport of KCl and/or NaCl into the tubule lumen, and by passive diffusion of substances. The hindgut reabsorbs essential metabolites, water and ions, mimicking the function of the distal tubular segments of the vertebrate nephron. As in the vertebrate kidney, the insect excretory system is regulated by neuroendocrine factors. These include both peptides and biogenic amines (Coast, 1996), (Spring, 1990). Diuretic peptides have been found in all insects studied so far. Their predominant action is stimulation of primary urine production by the Malpighian tubules (Gäde et al., 1997). In hematophagous insects the release of diuretic hormones is responsible for post-feeding diuresis (Gee, 1975), (Maddrell, 1991), (Wheelock et al., 1988). In xeric insects, where water preservation is of prime importance, diuretic hormones act both on primary urine production in Malpighian tubules and on the reabsorption of ions and water by the hindgut (Audsley et al., 1993). The result is an increased clearance of metabolic wastes from the hemolymph without incurring water loss. The latter peptides are therefore named "clearance factors" rather than diuretic factors (Nicolson, 1991).

It was shown previously that body extracts of *F. polyctena* contain diuretic factors (De Decker et al., 1994) and recently a diuretic peptide, which acts via cAMP, was purified from a head/thorax extract (Laenen et al., 1998). The present paper describes the purification and partial characterization of a second diuretic peptide from this ant species. The question of which signal transduction mechanism is involved, was also addressed.

MATERIALS AND METHODS

Insects

Ants (*F. polyctena*) were collected from their natural nest in a local forest (Slederlo (Genk), Belgium). They were reared at 25°C and 65% relative humidity under a 16h:8h light:dark cycle, and fed on a diet of sugar and water.

Purification

Approximately 300,000 ants were frozen instantly and kept in liquid nitrogen until further use. The frozen body parts could be separated by sieving.

Batches of the head/thorax fraction (40 g) were homogenized at 0°C in 120 ml of an ice-cold 10% TFA solution with a polytron (Heidolph Vax 600). The homogenate was left on ice for 10 min prior to centrifugation (14,000 rpm for 15 min). The pellet was washed twice with 120 ml of ice-cold 10% TFA.

Supernatants were collected and filtered in sequence over Whatman N°42 and Whatman N°1 filter paper prior to prepurification over home-made 4cc Vydac C_4 syringe cartridges (Varian). The cartridges were eluted stepwise with 90 ml of 0%, 20% , 40%, 65% and 80% CH_3CN in 0.1% TFA/water. The 0% CH_3CN fraction was discarded, the others were lyophilized in a Heto-Vac system and stored at -70 °C until further use.

Further purification was performed by means of reversed-phase high performance liquid chromatography (RP HPLC) on a Waters system consisting of two 510 pumps, a 486 tuneable absorbance detector set at 214 nm and the Millennium™ 2.15 chromatography manager. A first HPLC fractionation was performed on an analytical Waters Delta-Pak HPI C_{18} column (15µm, 300Å, 3.9x150 mm), using a linear gradient of 5 - 80% CH_3CN in 0.1% aqueous TFA over 60 min (flow rate : 1 ml/min). The peak fraction containing diuretic activity was lyophilized, resuspended in 200µl of 5% CH_3CN and loaded on a Pharmacia Sephasil Protein C_4 column (5µm, 300Å, 4.6x100mm). Final separation was obtained using a two-step linear gradient of CH_3CN, from 5 - 20% over 15 minutes and from 20% - 50% in 20 minutes, all in 0.1% aqueous TFA, with a 0.5 ml/min flow rate. Aliquots (containing 4 ant-eq.) of the obtained peak fractions were tested in fluid secretion assays on single isolated Malpighian tubules as described by De Decker *et al.* (1994). The purity of the active peak fraction from the Pharmacia Sephasil Protein C_4 column, was checked on a Phenomenex

Biosep Sec-S2000 column, using the following conditions. A linear gradient of CH_3CN, from 95 - 50% over 60 minutes in 0.01% aqueous TFA, with a 1.5 ml/min flow rate.

Experimental solutions

The composition of the insect saline was based on the composition of the ant's hemolymph (Van Kerkhove et al., 1989) and contained (in mmol/l) : 27.0 KCl, 8.04 K_3citrate, 1.97 $CaCl_2$, 13.0 $MgCl_2$, 16.8 Na_2fumarate, 14.4 Na_2succinate, 2.8 L-alanine, 10.5 trehalose, 11.7 maltose, 138.8 glucose and 12.1 Hepes. The saline had an osmolality of 350 mosm/kg and was adjusted to a pH of 7.2 by means of NaOH.

Chlorophenylthio-cyclic AMP (cpt-cAMP), A23187 and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma. Thapsigargin was purchased from Calbiochem. All substances (except IBMX and thapsigargin) were dissolved in the insect saline. IBMX and thapsigargin were prepared as stock solutions in DMSO (dimethyl sulfoxide : Across Chimica). Prior to use the stock solution was diluted with insect saline and the final concentration of DMSO was 2 $^{o}/_{oo}$ or less. The effect of 2 $^{o}/_{oo}$ DMSO was tested in control experiments. No significant effect was seen on the secretion rate or on the membrane potentials of single isolated Malpighian tubules. Concentrations used are specified in the Results section.

Measurement of membrane potentials

Membrane potentials of single isolated Malpighian tubules were measured according to the method described by Leyssens et al., (1992). Intracellular (Vbl) and transepithelial (Vte) membrane potentials were measured simultaneously using conventional microelectrodes (Borosilicate filament glass, Hilgenberg, Malsfeld, Germany; o.d. 1.3 mm, i.d. 0.7 mm) filled with 3 M KCl.

Determination of cAMP concentration

Isolated Malpighian tubules were preincubated for 15 min at room temperature in 25 µl of insect saline. Each test tube contained the isolated tubules of three ants or approximately 45 tubules (Van Kerkhove et al., 1989) . Phosphodiesterase activity was inhibited by adding IBMX to each of the test tubes (final concentration of 10^{-4}M). After a second incubation period of 15 min, 15 µl of insect saline with or without test sample was added to the tubes which were incubated for another 30 minutes. Addition of 900 µl of ice-cold methanol and

transfer to ice for 15 minutes stopped the reaction and precipitated the tissue proteins. The cAMP formed was liberated by sonication (3 times 30 seconds). The samples were centrifuged (13,000 rpm for 15 min), the supernatant was lyophilized and its cAMP content determined by means of a ^{125}I cAMP scintillation proximity assay kit, according to the manufacturer's instructions (Amersham International, RPA538). The protein content of the pellets was determined by means of a BCA-protein determination kit (Pierce) with bovine serum albumin (BSA) as a standard.

Mass spectrometry and amino terminal sequencing

The mass of the purified peptide was determined on a Micromass TofSpec Matrix Assisted Laser Desorption Ionisation - Time Of Flight (MALDI-TOF) mass spectrometer. A 1 µl aliquot of the HPLC fraction was mixed with an equal amount of a 1:1 solution of CH_3CN - MetOH containing 50 mM α–cyanohydroxycinnamic acid. One µl of this mix was spotted onto a stainless steel target plate, which was introduced into the mass spectrometer and analysed. Laser intensity was adjusted to near threshold values to obtain best resolution. The instrument was operated in reflectron mode (accelerating voltage 24 kV, reflector voltage 29 kV). Typically 20 - 25 shots were averaged to obtain the final spectrum. Amino acid sequence information was obtained by Edman degradation on a Beckman (Model LF 3600 TC) gas phase sequencer, equipped with an on line PTH amino acid analyser (Beckman System Gold Model 168 DAD).

Statistics

Results are given as mean values ± standard error (S.E.M.), with the number of tubules in parentheses. Statistical significance was calculated using the paired Student's *t*-test, unless indicated otherwise. A difference was considered significant if $p \leq 0.05$.

RESULTS

Purification

Prepurification of the crude head extract resulted in four subfractions characterized by a stepwise increase in hydrophobicity. All fractions were tested in the fluid secretion assay on single isolated Malpighian tubules of the ant. At a concentration of 0.1 ant-eq/µl the 20% CH_3CN fraction stimulated the fluid secretion rate to 220% ± 21 (n=6, $p \leq 0.05$) of the basal rate (Fig. 3.1A). Upon washout of the extract a small additional increase to 227% ± 16 (n=6, $p \leq 0.05$) was observed. The stimulation was sustained throughout the rest of the experiment. The effect of the 40% CH_3CN fraction (Fig. 3.1B) was clearly different : a small diuretic effect to 182% ± 28 (n=5, $p \leq 0.05$), was seen during the test period, which was completely abolished during the washout period. The diuretic effect was lost in the 65% CH_3CN fraction (Fig. 3.1C). At a concentration of 0.1 ant-eq/µl the secretion rate was reduced to 75% ± 5 (n=5, $p \leq 0.05$) during the test period with a full recovery on washout of the extract. The 80% CH_3CN fraction (Fig. 3.1D) had no significant effect throughout the experiment.

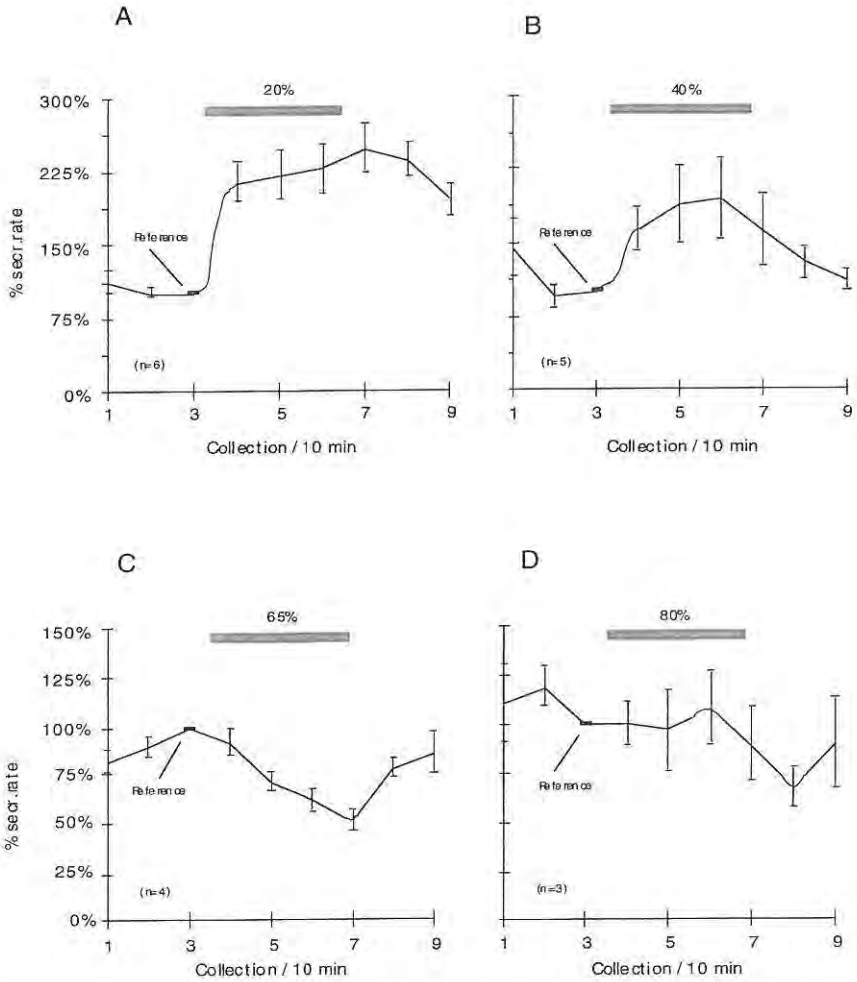

Figure 3.1. Effect of different CH_3CN fractions on fluid secretion by isolated Malpighian tubules of the forest ant *Formica polyctena*. All fractions were tested at a concentration of 0.1 ant-eq / µl. The presence of the fractions in the bath, is indicated by the bars. Values represent mean ± S.E.M., (n=number of tubules tested).

Based on the clear and sustained diuretic effect of the 20% CH_3CN fraction we concluded that this fraction might contain one or more diuretic factors. It was used for further chromatographic purification.

In a first experiment a small amount (4 ant-eq) of the 20% CH_3CN fraction was loaded on the Waters Delta-Pak HPI C_{18} column. Of the 4 UV-absorbing peaks (Fig. 3.2), which were collected manually, only one fraction, eluting between 23.9 and 24.6 min at 26% CH_3CN, stimulated the fluid secretion. The peak value was 245% ± 29 (n=4, $p \leq 0.05$) when tested at a concentration of 0.1 ant-eq/µl. None of the other fractions had a significant effect ($p > 0.05$).

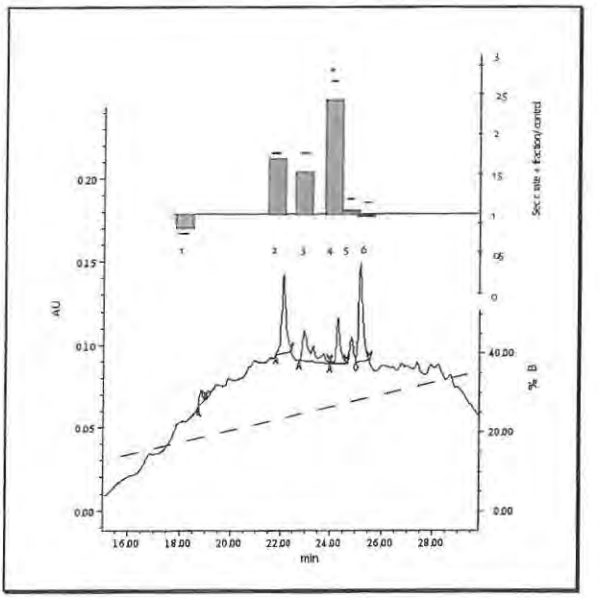

Figure 3.2. UV absorbance profile of 4 ant-eq of the 20% SepPak fraction separated on the Waters Delta-Pak HPI C18 column (300Å, 3.9x150mm). Conditions are described in Materials and Methods. The dashed line represents the concentration of solvent B. The upper panel shows the effect of the tested peak fractions (mean values ± S.E.M., n = 6 tubules) in the fluid secretion assay tested at 0.1 ant-eq / µl. * Significantly different from 1 ($p \leq 0.05$, paired Student's *t*-test).

In a subsequent experiment, 100 ant-eq of the 20% CH_3CN fraction were loaded on the same column. Based on the results of the first run with 4 ant-eq, the fraction containing the diuretic activity was collected based on its retention time and UV-absorbance profile. Fop-DP eluted between 23.7 and 24.5 min. at 26% CH_3CN. Tested at a concentration of 0.1 ant-eq/µl, it doubled the fluid secretion rate to 222% ± 18 (n=8, p 0.05).

The bioactive fraction was lyophilized, redissolved in 200µl of 5% CH_3CN and loaded on a Pharmacia Sephasil Protein C4 column (Fig. 3.3). Fop-DP eluted at 33% CH_3CN and it doubled the fluid secretion rate to 218% ± 11 (n=6, p 0.05), when tested at a concentration of 0.1 ant-eq/µl.

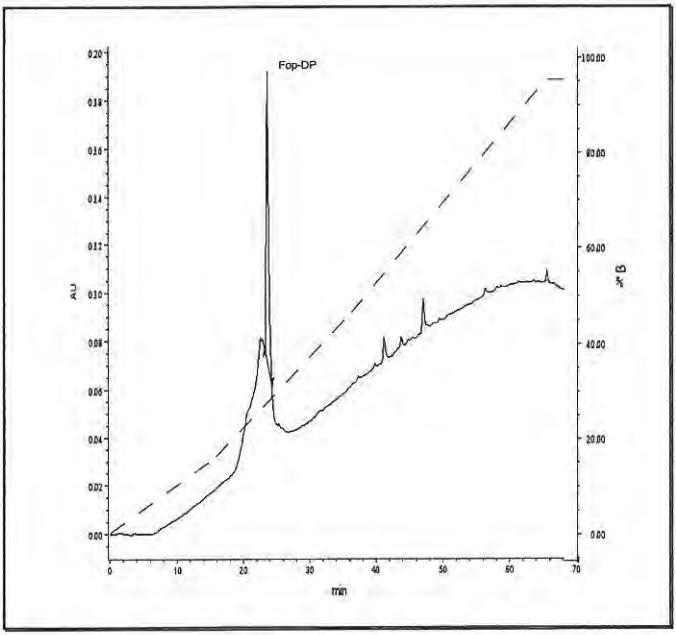

Figure 3.3. UV absorbance profile of 100 ant-eq of Fop-DP separated on the Pharmacia Sephasil Protein C4 column (300Å, 4.6x300mm). Conditions are described in Materials and Methods. The dashed line represents the concentration of solvent B.

Finally, Fop-DP was run on a Phenomenex Biosep Sec-S2000 column and eluted in a single UV-absorbing peak at 42% CH_3CN (data not shown). It may therefore be assumed that Fop-DP was already pure after the fractionation on the Pharmacia Sephasil Protein C_4 column. The stimulation of fluid secretion was still present in the peak fraction but, due to the loss of material, the test concentration in the fluid secretion assay had to be increased to a value of 2.0 ant-eq/µl in order to obtain a stimulation in the fluid secretion experiments to a value of 138% ± 4 (n=6, $p \leq 0.05$). Indeed, the major disadvantage of the Biosep column is the low yield. Most of the loaded material comes off in the injection peak, only a very small fraction is retained and recovered on elution. For further analysis of the mode of action and the molecular characterization we worked with Fop-DP purified up to the C_4 level, but instead of 100 ant-eq, 10,000 ant-eq of the 20% CH_3CN fraction were loaded on the analytical C_{18} column. In total 28 runs were needed to process the total crude extract. Since most of this material was used to study the mode of action of Fop-DP, insufficient material was left for the molecular characterization.

Biological responses of isolated Malpighian tubules of the ant to Fop-DP

Based on the initial sequencing yields obtained in the first Edman cycle, we calculated that at least 67 pmol was purified from 90 ants. Neglecting loss of material during purification, this would suggest that 1 ant contains at least 0.74 pmol of the diuretic peptide.

When Fop-DP was used at a physiological concentration (0.01 ant-eq/µl corresponds to 7.4 nM based on peptide recovery), the stimulation of fluid secretion was even more pronounced than at the 0.1 ant-eq/µl dose applied in the bioassays during purification. This concentration of 7.4 nM tripled the fluid secretion rate to 375% ± 23 (n=8, $p \leq 0.05$) (Fig. 3.4).

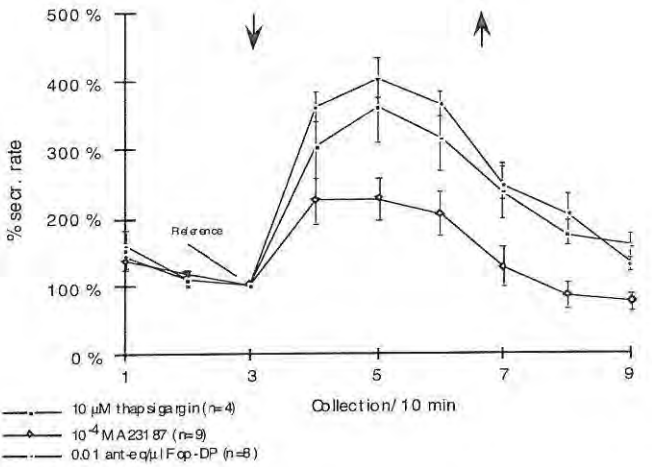

Figure 3.4. Effect of different test substances on fluid secretion by isolated Malpighian tubules of the forest ant *Formica polyctena*. Arrows indicate addition (↓) or removal (↑) of the test substances. Values represent mean ± S.E.M., (n=number of tubules tested).

At a concentration of 0.1 ant-eq/µl, Fop-DP had no effect on the basolateral membrane potential but reversibly depolarized the transepithelial potential by 18.3 mV ± 3.7 (n=4) (Fig. 3.5). Therefore, the transepithelial depolarization is due to a depolarization of the apical membrane potential.

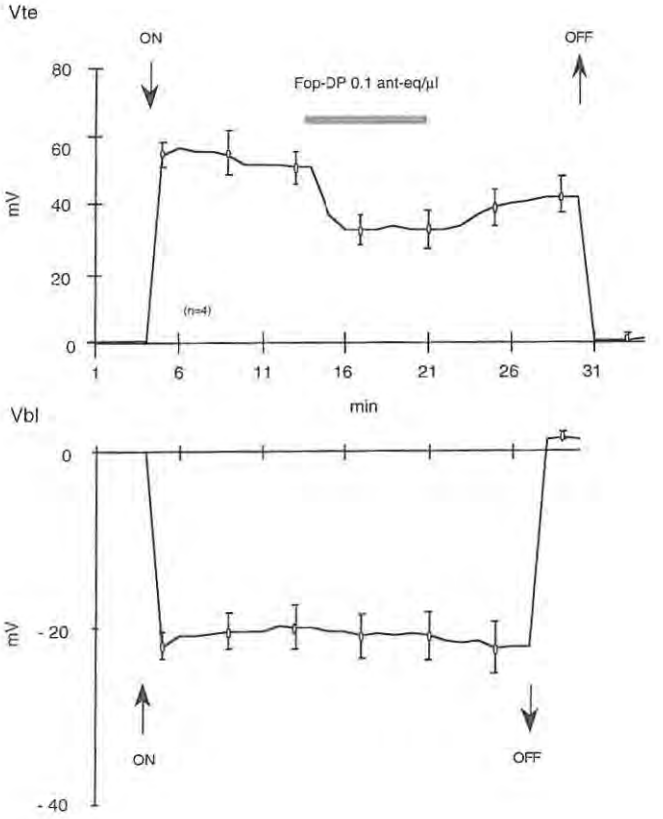

Figure 3.5. Running mean for the effect of Fop-DP tested at a concentration of 0.1 ant-eq /µl on the transepithelial (Vte) and the intracellular potential (Vbl) measured simultaneously in single isolated Malpighian tubules. Values represent mean ± S.E.M., (n=number of tubules tested). Arrows indicate impalement (ON) or withdrawal (OFF) of the microelectrodes.

Intracellular cAMP measurements

From earlier experiments (De Decker, 1993) we knew that 10µM cpt-cAMP doubled the fluid secretion rate in single isolated Malpighian tubules. A similar response is obtained with 0.1 ant-eq/µl of Fop-DP. To find out whether Fop-DP acts via cAMP as a second messenger, the effect of the peptide on the intracellular cAMP levels was measured.

The responsiveness of the tubules to 0.1 ant-eq/µl of the 40% CH_3CN fraction, known to contain *F. polyctena* cAMP generating peptide (Laenen et al., 1998), was taken as a positive control.

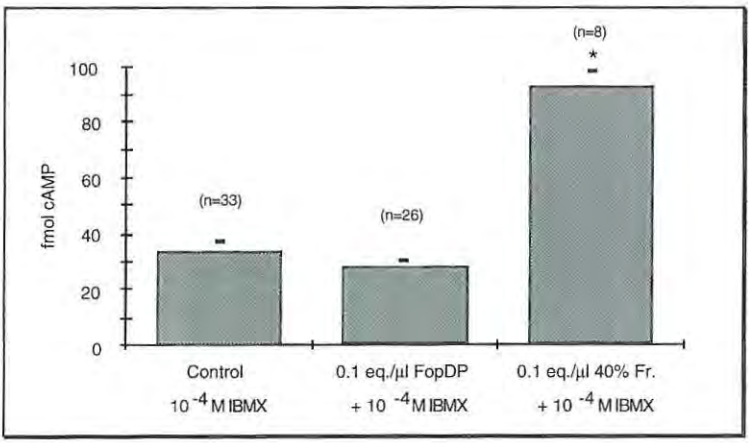

Figure 3.6. cAMP production in the Malpighian tubules of the ant in different test conditions. Mean values ± S.E.M., (n=number of tubules tested). * Significantly different from the control in 10^{-4} M IBMX , unpaired Student's *t*-test, $p \leq 0.05$. The response to 0.1 ant-eq/µl of the 40% CH_3CN fraction, known to contain *F. polyctena* cAMP generating peptide (Laenen et al., 1998), was taken as a positive control.

These results (Fig. 3.6) show that Fop-DP had no effect (28 fmol ± 2, n=26) on the cAMP levels when compared to the effect of 10^{-4}M IBMX (33 fmol ± 3, n=36) alone. The 40% CH_3CN fraction on the other hand tripled the cAMP level to an absolute value of 93 fmol ± 6 (n=8). The tubules of three ants (i.e. approximately 45 tubules see Van Kerkhove et al., 1989) were processed in each test tube. The cAMP content per tubule can therefore be estimated to be 0.73 fmol / tubule ± 0.07 in control conditions or 0.62 fmol / tubule ± 0.04 in the presence of Fop-DP. It increases to 2.07 fmol / tubule ± 0.13 in the presence of the 40% CH_3CN fraction.

Effect of A23187 on the Malpighian tubules of the ant

A calcium ionophore A23187 (Sigma) was used to increase the intracellular calcium concentration in the isolated Malpighian tubule. A test concentration of 10^{-4} M was used since according to De Decker (1993) it is the lowest concentration with a significant effect on fluid secretion in ant's tubules.

At this concentration the calcium ionophore mimicked the effect of Fop-DP on primary urine production. It doubled the fluid secretion rate to 218% ± 31 (n=9, $p \leq 0.05$) (Fig. 3.4) and after washout, the fluid secretion rate returned to the control value. At the same test concentration, A23187 depolarised the transepithelial membrane potential from 55 mV ± 8 to 44 mV ± 5 (n=6) (Fig. 3.7).

Like the diuretic peptide, the calcium ionophore had no effect on the basolateral membrane potential. The effect of Fop-DP on V_{te} recovered partially (Fig. 3.5), that of A23187 was less reversible. The reason for this is not clear, but might be due to the fact that the washout period is much shorter, compared to the fluid secretion experiments and that removal of the lipophilic ionophore requires more time. Even for the fluid secretion experiments not all secretion rates fell to the control value within the first 10 min. of the washout period.

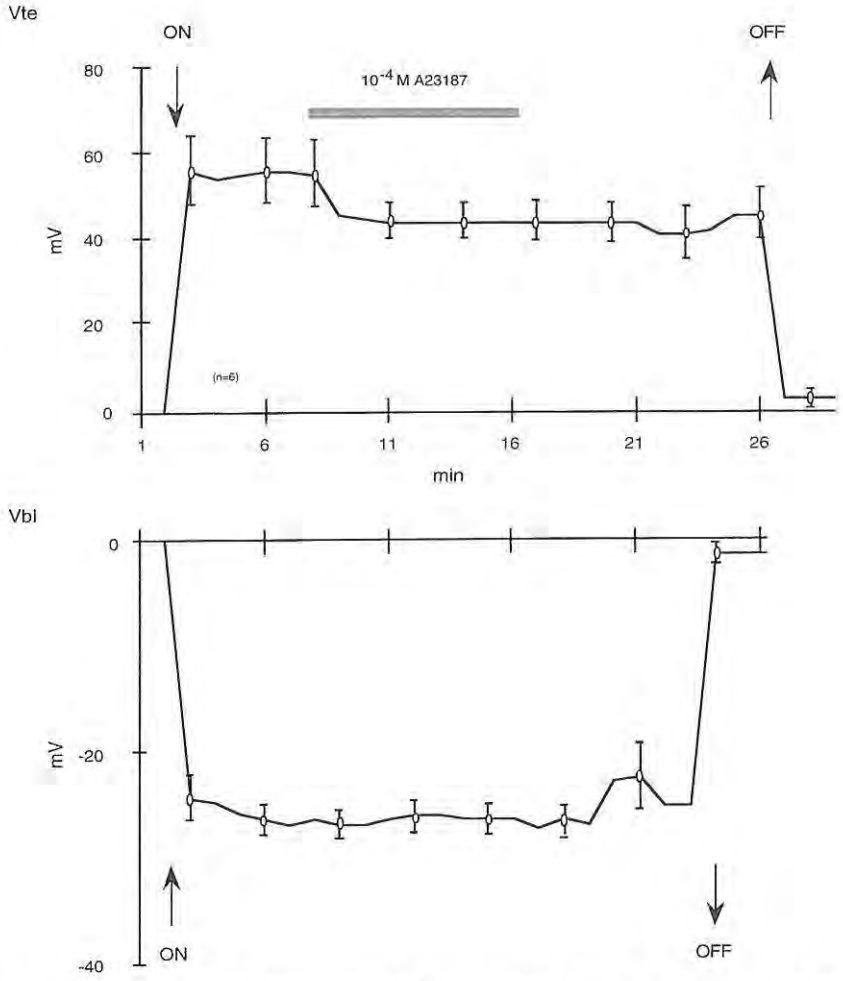

Figure 3.7. Running mean for the effect of ionophore A23187 tested at a concentration of 10^{-4}M on the transepithelial (Vte) and the intracellular potential (Vbl) measured simultaneously in single isolated Malpighian tubules. Values represent mean ± S.E.M., (n=number of tubules tested). Arrows indicate impalement (ON) or withdrawal (OFF) of the microelectrodes.

Similarity in the effects of Fop-DP and 10μM thapsigargin

To assess the involvement of the release of calcium from intracellular stores as a second messenger for the diuretic activity of Fop-DP, thapsigargin, a Ca^{2+}-ATPase inhibitor that prevents reuptake of Ca^{2+} into intracellular stores, was used. At a test concentration of 10 μM, thapsigargin tripled the fluid secretion rate to 327% ± 60 (n=4, $p \leq 0.05$). Subsequently 0.1 ant-eq/μl of Fop-DP and 10 μM of thapsigargin were tested separately and then together on the same tubule. These experiments revealed that Fop-DP had no additive effect on the stimulation caused by thapsigargin or vice versa (Fig. 3.8A). The involvement of calcium was tested further in another set of experiments. In this series 10μM cpt-cAMP was added to the same tubule after stimulation with either 0.1 ant-eq/μl of Fop-DP or 10 μM of thapsigargin. In both cases a clear additive effect of cpt-cAMP to the first stimulus was observed (Fig. 3.8B). Apparently Fop-DP and thapsigargin stimulate fluid secretion by ant tubules via a cyclic-AMP independent mechanism.

Molecular characterization of Fop-DP

Mass spectrometry on a sample of 10 ant-eq confirmed the purity of Fop-DP and revealed a mass of 7,514 Da (Fig. 3.9). This is much larger than any of the diuretic peptides known to date and comparable to the size of the small prothoracicotropic hormones (PTTH) formerly known as insect activating hormones (Watson et al., 1989). So far 25 amino acids (VPKYENCVSEVLPAGDRQRCVKVTC) have been determined by microsequencing on a sample of 90 ant-eq. No further sequence could be read due to a typical fading PTH amino acid signal in prolonged Edman degradation runs. A full sequence of the peptide could not be obtained due to lack of material, but the sequence obtained so far is characterized by the presence of three cysteine residues (underlined).

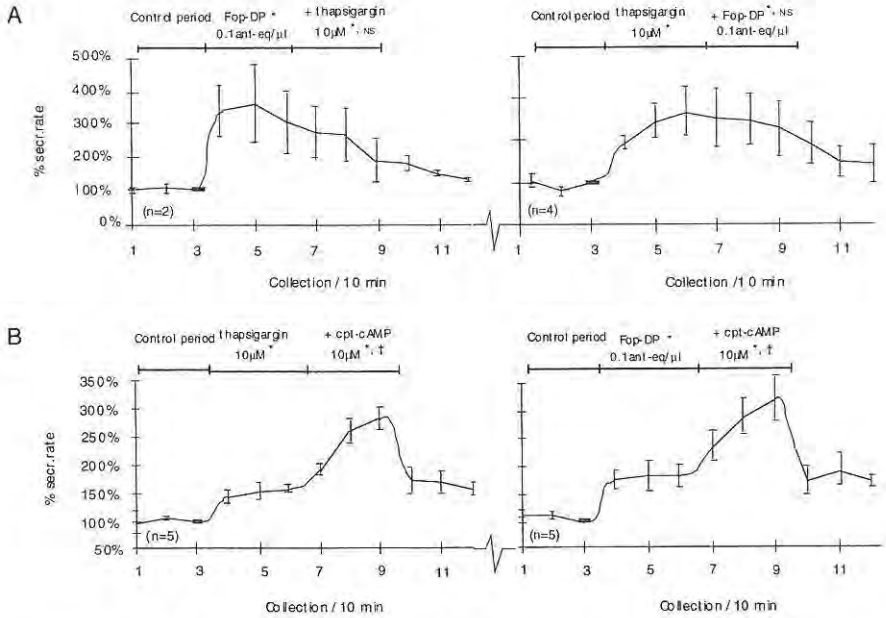

Figure 3.8. Effect of Fop-DP, thapsigargin and cpt-cAMP on fluid secretion by isolated Malpighian tubules of the forest ant Formica polyctena. The presence of the agents in the bath is indicated by the bars. A. Thapsigargin has no additive effect on the stimulation of the fluid secretion with Fop-DP or vice versa. B. An additive effect of cpt-cAMP to both Fop-DP or thapsigargin is evident. * Significantly different from the control period ($p \leq 0.05$, paired Student's t-test). † Significantly different from the first test period ($p < 0.05$, paired Student's t-test). NS not significantly different from the first test period. Values represent mean ± S.E.M., (n=number of tubules tested).

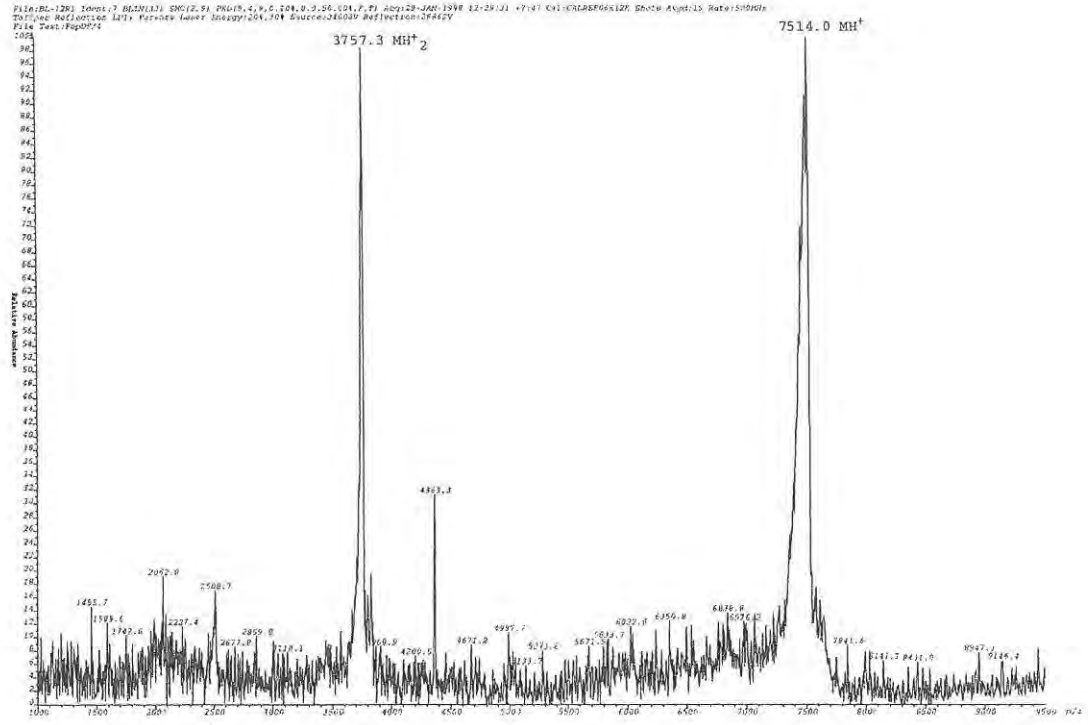

Figure 3.9. Mass spectrum obtained for 10 ant-eq of the C4 fraction. Only one substance was present. The first peak is the double charged ion, MH+2, of the native peptide, sensed by the system as having only half of the actual mass.

DISCUSSION

Purification and biological activity

A diuretic peptide was purified from a 10% TFA head/thorax extract of the forest ant *F. polyctena* by means of a two-step reversed-phase HPLC-protocol. It was named '*F. polyctena* diuretic peptide' and tripled the fluid secretion rate when tested at an estimated concentration of 7.4 nM. This concentration is of the same order of magnitude as that reported for *Periplaneta* DP on cockroach tubules (1-10 nM) (Kay et al., 1992), for *Musca* DP on *Manduca* tubules (1-3 nM) (Spittaels et al., 1996) and for CRF-related peptides on cAMP production in *Manduca* Malpighian tubules (5-9.3 nM) (Audsley et al., 1995). The effectiveness of Fop-DP in the nanomolar range, which is the range in which many hormones exert their physiological effect, supports the idea that Fop-DP is a major diuretic factor in the forest ant. Fop-DP stimulated primary urine production and reversibly depolarized the apical membrane potential. Considering the diuretic effect of Fop-DP and the transport model for the ant's Malpighian tubule (Leyssens et al., 1994; Van Kerkhove, 1994), the following working hypothesis is proposed. Fop-DP may induce the increase of an apical chloride conductance. A transcellular chloride conductance in the ant's Malpighian tubules has been demonstrated with the measurement of changes in intracellular chloride concentrations in response to changes in bath chloride concentration (Dijkstra et al., 1994). The increase of an apical chloride conductance will depolarize the apical membrane potential and as such reduce the electrochemical gradient against which the proton pump provides protons for the apical K^+/H^+-antiporter (Zhang et al., 1994). As a consequence, the pumping rate and K^+ extrusion rate can accelerate, accompanied by an increase in fluid secretion rate. Further experiments using specific chloride channel blockers should be performed to decipher this depolarization of the apical membrane potential in the presence of Fop-DP.

Molecular characteristics

A remarkable characteristic of the peptide sequence obtained so far is the presence of three cysteine residues. This suggests that Fop-DP is a cysteine-rich peptide in which multiple cysteine bridges might be important for the formation of the three-dimensional structure of the peptide. The importance of disulphide bridges in relation to receptor-binding characteristics has been well studied for other cysteine-rich peptides such as the scorpion α-toxins and fungal

toxins (Gu et al., 1996), (Housset and Fontecilla-Camps, 1996). From these studies it is clear that besides binding characteristics, peptide stability is also related to the three-dimensional folding pattern (Li et al., 1996), (Loret et al., 1990). All these cysteine-rich peptides are very stable, resisting organic solvents, elevated temperatures and digestion by proteolytic enzymes. The explanation for this stability is in part due to embedding of the cleavage sites in the inner core of the peptide structure (Gu et al., 1996).

In insects the cysteine-rich neuropeptides can be classified in four subfamilies: the ecdysiotropic hormone (PTTH) family known to regulate the biosynthesis of ecdysteroids in the prothoracic glands (Watson et al., 1989), the bombyxins isolated from the brains of *Bombyx mori* , having a structural relation to the vertebrate insulins (Nagasawa et al., 1988), the ion transport peptide (ITP) family, known to stimulate salt and water reabsorption and inhibit acid secretion in the ileum of locusts (Audsley et al., 1992) and finally the peptide family of neuroparsins, known to stimulate fluid reabsorption in the rectal complex of insects (Girardie and Fournier, 1993). For the bombyxins, structure-function studies revealed that residues responsible for the formation of a hydrophobic core at the centre of the molecule are highly conserved, whereas the surface residues differ from each other (Nagasawa et al., 1988).

The burial of hydrophobic residues at the inside of the three-dimensional structure can explain the elution behaviour of Fop-DP in the HPLC-purification protocol. Although we are dealing with a large (>7,000 Da) peptide compared to the insect diuretic peptides obtained so far, it elutes at 26 - 35% of CH_3CN from the reversed-phase HPLC-columns. This elution in a hydrophilic solvent is similar to the behaviour observed for the scorpion and fungal toxins (De Dianous et al., 1987),(Ji et al., 1988), (Kopeyan et al., 1990), (Loret et al., 1990). A computer search of sequence data banks did not reveal any significant similarity of Fop-DP with other known cysteine rich peptides. Possibly, similarities in Cys residue distribution may become apparent only after total identification of the amino acid sequence.

Signal transduction pathway

From the ^{125}I cAMP scintillation proximity assay it was clear that Fop-DP had no effect on intracellular cAMP concentrations. The calcium ionophore A23187 and the calcium metabolizing drug thapsigargin, on the other hand, mimicked the effect of Fop-DP on primary

urine production. This suggests that Fop-DP uses an increase of intracellular calcium as a signal transduction mechanism. We therefore tried to measure the intracellular Ca^{2+} activity with the fluorescent dye FURA-2. However, the dye was secreted into the lumen and the remaining intracellular dye concentration was too low to measure intracellular calcium changes (unpublished results). Similar problems with micro-fluorescence technique have been reported for the Malpighian tubules of *Drosophila* (Rosay et al., 1997).
The observed diuretic effect was realised via a cAMP independent mechanism as shown by the additive effect of the cell permeant cAMP analogue, cpt-cAMP. Dual control of insect Malpighian tubules has previously been reported for *Locusta* (Coast, 1995), *Aedes* (Ripoll et al., 1996) and *Drosophila* (Davies et al., 1995). In all of these insects two peptides or a peptide and a biogenic amine act together, via separate second messengers, to obtain a maximal stimulation of the primary urine production. For the forest ant this second peptide might be the cAMP generating peptide FopcGP, recently purified from the 40% CH_3CN fraction from a 10% TFA head/thorax extract (Laenen et al., 1998). A dual control would have a number of advantages. Less material would be needed to get an effect on primary urine production and as a consequence less energy would be required since peptide synthesis is metabolically expensive, especially for larger peptides like Fop-DP. A second advantage has to do with improved tuning. A faster and finer regulation can be obtained with two peptides (Coast, 1996). Whether this situation exists in the ant will be checked in future experiments by applying both FopDP and FopcGP at submaximal concentrations.

In conclusion, a diuretic peptide, Fop-DP, was purified from the 20% CH_3CN fraction of a 10%TFA head/thorax extract of the forest ant *F. polyctena*. Fop-DP has a molecular mass of 7,514 daltons, which is much larger than all known insect diuretic peptides. The N-terminal sequence obtained is completely different from those of all the diuretic peptides known so far in insects. It is a cysteine-rich peptide, suggesting the importance of a three dimensional scaffold created by disulphide bridges. The biological effect of Fop-DP could be mimicked with A23187, a calcium ionophore, and by thapsigargin, an inhibitor of the endoplasmic reticulum calcium ATPase, which indicates that Fop-DP acts via an increase in intracellular calcium.

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CHAPTER 4

Diuretic and antidiuretic factors in body extracts from the forest ant and the honeybee: a chromatographic comparison of factors affecting the ant Malpighian tubule

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Running head : Diuretic and antidiuretic factors in body extracts from Hymenoptera

ABSTRACT

Crude trifluoroacetic acid (TFA) extracts were made from different body parts of the forest ant and the honeybee. The crude extracts were prepurified by means of solid phase extraction over reversed-phase cartridges, and the resulting prepurified fractions were studied for their effect on the fluid secretion in single isolated Malpighian tubules of the ant. Diuretic and antidiuretic factors were shown to be present. High performance liquid chromatography allowed us to identify a diuretic factor from a head extract of the bee (Apm-DF) which had a similar elution behaviour as the previously purified diuretic peptide from head extracts of the ant (Fop-DP). Likewise an antidiuretic factor (ApmADF) was identified in the bee's abdomen extract. It inhibited fluid secretion and depolarized the transepithelial potential difference in the same way as FopADF, the antidiuretic factor purified from the ant's abdomen extract.

Keywords: insect, Hymenoptera, primary urine production

INTRODUCTION

Species belonging to the same insect family or even the same insect order have been shown to possess low molecular weight proteins and polypeptides with similar properties. Comparative studies on the protein composition of hymenopteran venom reservoirs for instance showed that low molecular weight proteins and polypeptides are typical for the venoms of ants, social wasps and bees (Schmidt et al., 1986). Similar enzymatic activities were detected for the venoms of nine ant species and nine species of social wasps, suggesting that similar factors were conserved in the hymenopteran venoms. So in this respect, ants are linked to bees and social wasps in the same phylogenetic tree (Leluk et al., 1989). Conversely an evolutionary relationship has also been used to identify peptides: in cockroaches for instance the approach was used for the identification of the hypertrehalosaemic peptides (Gäde, 1989). Gland extracts were made of the corpora cardiaca of 14 different Blattaria species and chromatographically compared to identified peptides of the American cockroach *Periplaneta americana*. Peptides with identical, or almost identical properties were identified in all of the investigated species. Another example is the identification of adipokinetic hormones (AKH) in methanol extracts of the corpora cardiaca of various dung beetles where this approach was used succesfully (Gäde, 1997). Six species were investigated and in all of them two forms of AKH family peptides could be identified based on their retention time on an analytical C_{18} column.

It seemed therefore interesting to find out whether similar endogenous diuretic and antidiuretic peptides were present in the ant and the honeybee (*Apis mellifera carnica*). The honeybee is available in large numbers and it is from the same insect order as the forest ant. Furthermore it is a large insect, providing more material.

In a previous study we tried to characterize an antidiuretic factor from a 15% TFA abdomen extract of the forest ant *Formica polyctena*. (Laenen et al., 1998a). We encountered the problem that an extract made of 150,000 ants did not yield enough material for the molecular characterization of the factor. In order to obtain a sufficient amount of an insect neuropeptide for structural characterization, processing of gram quantities of tissue extracts may be needed (Holman et al., 1990). This is time consuming for the ant: the collection of 150,000 ants took three weeks. Bees are much larger and as such it should be possible to make body extracts from a small number of insects each individual having a much higher peptide content. As they are phylogenetically related to the ant, the presence of similar factors in the tissue extracts of both insect species is to be expected.

In this study we first of all tried to corroborate this hypothesis and to establish the presence of factors in body extracts of the bee that have properties comparable to those found in the ant. According to the results both insect species contain similar factors which could be chromatograpically identified and which had a comparable effect on Malpighian tubules of the ant.

MATERIALS AND METHODS

Insects

The ants used in the experiments were collected from their natural nest in the forest of Slederlo (Genk, Belgium). They were reared at 25°C and 65% relative humidity under a 16h:8h light:dark cycle, and fed a diet of sugar and water. Honeybees were obtained from a local bee-keeper.

Saline

The composition of the insect saline was based on the composition of the ant hemolymph (Van Kerkhove et al., 1989) and contained (in mmol/l) : 27.0 KCl, 8.04 K_3citrate, 1.97 $CaCl_2$, 13.0 $MgCl_2$, 16.8 Na_2fumarate, 14.4 Na_2succinate, 2.80 L-alanine, 10.5 trehalose, 11.7 maltose, 138.8 glucose and 12.1 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (Hepes). The saline had an osmolality of 350 mosm/kg and was adjusted to a pH of 7.2 by means of NaOH.

Preparation of a 15% TFA bee's abdomen extract

Approximately 200 bees were collected from a bee-hive of a local bee-keeper (Alken, Belgium). The bees were placed in a freezer at -20°C for 15 minutes. Subsequently the different body parts were separated manually and stored in liquid nitrogen until further use. Approximately 15,000 ants were collected from their natural nest in Slederlo (Genk, Belgium). They were frozen instantly and kept in liquid nitrogen until further use. The frozen body parts were separated from one another by sieving. The abdomina were retained with a 1.7 mm sieve, the heads and thoraces with a 1.4 mm sieve. The separated body parts were kept in liquid nitrogen until further use.

Three batches of 65 bee's abdomina (5 g) or five batches of 200 ant's abdomina (2 g) were transferred into 30 ml centrifugation tubes. They were crushed manually by means of a pestle and 25 ml of an icecold 15 % TFA solution was added. This mixture was shaken

vigourously and left on ice for 10 min prior to centrifugation. The bottles were centrifuged in a Beckmann JA20 rotor at 14,000 rpm for 15 min. The supernatant was collected and the pellet washed twice with 25 ml of icecold 15% TFA. The supernatant was filtered in sequence over Whatman N°42 and Whatman N°1 filter paper prior to prepurification over 35cc C18 Cartridges (SepPak Vac, Waters). The cartridges had previously been wetted with acetonitrile (CH_3CN) and were equilibrated in milliQ (Millipore) water. The cartridges were eluted stepwise with 90 ml of 0%, 20% , 40%, 65% and 80% CH_3CN in 0.1% TFA/water. The fractions were lyophilised separately in a Heto-Vac system and stored in the freezer at -70 °C until further use.

Preparation of 10% TFA head extracts.

Approximately 1,500 bees were collected from the bee-hive by tapping the hive-frames, containing the honeycombs, above a container filled with liquid nitrogen. The bees were frozen instantly. The different body parts were separated manually and stored in the freezer at - 70°C until further use.

The heads and thoraces from all 15,000 ants collected (see above) were used to prepare the head / thorax extract of the ants.

Batches of 500 bee heads (10g) or of 1000 ant-eq of the head / thorax mixture (5g) were transferred into a series of 30 ml centrifugation tubes, containing 25 ml of an ice-cold 10 % TFA solution as extraction solvent.

The tubes were kept on ice and their content was homogenised by means of a polytron (Heidolph Vax 600) for 3 minutes. Further treatment of the homogenate was as for the bee's and ant's abdomen extract (see above). Except for the prepurification, instead of the 35cc C_{18} cartridges (SepPak Vac, Waters), custom made 4cc Vydac C_4 syringe cartridges (Varian) were used.

Protein determinations

Small aliquots (1-2 ml) were taken from the crude tissue extracts and lyophilised by means of a Hetovac VR-1. In order to resuspend the dried protein pellets 50 µl of 3M urea was added, the samples were vortexed and kept at 60°C for 30 min. After this incubation the samples were sonicated (2x30s), pulse centrifuged and the protein content was determined in the BCA protein assay reagent kit from Pierce, according to the instructions supplied by the manufacturer.

Chromatographic comparison

The prepurified fractions were chromatographically analysed by means of reversed-phase high performance liquid chromatography (RP HPLC) on a Waters system consisting of two 510 pumps, a 486 tunable absorbance detector set at 214 nm and the Millennium 2.15 chromatography manager. Appropriate amounts of the different prepurified fractions (see Results section) were loaded on an analytical Waters Delta-Pak HPI C18 (5µm, 300Å, 3.9x150 mm) column. The column was eluted with a linear gradient of 5 - 80% CH_3CN in constant 0.1% aqueous TFA over 50 min, with a flow rate of 1ml/min. Peak fractions were collected manually. Samples of these fractions were dried in polypropylene eppendorf tubes by centrifugal evaporation. They were redissolved in insect saline and their biological effects were tested in the different bioassays as described below.

Fluid secretion experiments

Fluid secretion experiments were performed on the Malpighian tubule of the ant as described previously (De Decker et al., 1994). Briefly, an ant was dissected in a small petri-dish in fresh saline. The midgut with the Malpighian tubules still attached to it was transferred to another petri-dish containing fresh saline. A Malpighian tubule was cut free and transferred into a petri-dish in a 50 µl bathing droplet, covered with paraffin oil. The cut end of the Malpighian tubule was fixed into a holding pipette and pulled out into the paraffin oil. To release the primary urine produced, the tubule was nicked by the tip of a collecting pipette (borosilicate filament glass, Hilgenberg, Malsfeld, Germany; o.d. 1.3 mm, i.d. 0.7 mm). The primary urine collected during the first 10 min was discarded. Secreted fluid was subsequently collected and the volume measured every 10 minutes. The bath was perfused with insect saline, at a rate of 200 µl/min.

A fluid secretion experiment consisted of three 30 minute periods , each consisting of three collections of 10 minutes each. The first period was a control period of which the third secretion droplet was taken as a reference. During the second period or test period, the bath perfusion was switched off and the bathing droplet was replaced with a test solution of 50 µl. In the final period the bath perfusion was switched on again and the test solution was washed out and replaced with control saline. Results were expressed as a percentage of the internal reference. Therefore each tubule served as its own control.

Measurement of membrane potentials

The measurement of membrane potentials was adapted from a method described by Leyssens *et al.* (1992). A single Malpighian tubule, freshly dissected from an ant, was

immobilized by means of two holding pipettes, at the bottom of a 50 µl bathing droplet, covered with paraffin oil. This immobilisation was essential to prevent tubule movement due to the continuous perfusion of the bath with insect saline.

The transepithelial (V_{te}) as well as the basolateral membrane (V_{bl}) potential was measured, using conventional, high resistance (20 - 40 MΩ) microelectrodes (Borosilicate filament glass, Hilgenberg, FRG; OD 1.5 mm, ID 1 mm; tip diameter < 0.5 µm). They were filled with 3M KCl and connected to a dual probe electrometer (WPI Model M750) via a Ag/AgCl wire. A low resistance (1 MΩ) reference electrode (filled with 3M KCl) was connected to the ground via a Ag/AgCl wire and closed the electrical circuit. The simultaneous measurement of both potentials was set up following the procedure described below.

The first microelectrode was pushed through the cell layer into the lumen and measured V_{te}. Subsequently a cell was impaled with the second microelectrode in order to measure V_{bl}. The impalement was accepted if a sudden voltage deflection occurred that remained stable for at least a few minutes. The results were recorded on a dual-pen recorder (Philips Model PMB 8252). They were accepted only if the microelectrode potential differed not more than ± 4 mV from zero after withdrawal of the microelectrode at the end of the experiment. The apical membrane potential (V_{ap}) was calculated as the difference between V_{bl} and V_{te} ($V_{ap} = V_{bl} - V_{te}$). Each experiment consisted of three periods of 10 - 15 minutes. After a control period of 10 - 15 minutes, the bath perfusion was stopped, the bathing droplet removed and the test solution added. Stopping the bath perfusion on its own did not affect the membrane potentials as seen in a set of ten control experiments (results not shown). Finally the test solution was washed out by a continuous perfusion with insect saline.

Statistics

Results are given as mean values ± standard error (S.E.M.), with the number of tubules in parentheses. Statistical significance was calculated using the paired Student's *t*-test, unless indicated otherwise. A difference was considered significant if $p \leq 0.05$.

RESULTS

Comparison of the peptide content of the abdomen extracts

One and a half ml of the crude 15% TFA abdomen extract of the ant was used in the BCA protein determination kit using bovine serum albumine (BSA) as standard. Expressed as µg BSA equivalents / ant-eq this extract yielded 26.2 µg/ant-eq ± 4.4 (n=5). This yield was compared to the 15% TFA abdomen extract of the honeybee. A sample of 1.8 ml of this extract was tested and yielded a protein content of 114 µg/bee-eq ± 23 (n=8).
The protein content of the 10% TFA head extracts was not determined.

Effects of crude 15% TFA abdomen extracts on fluid secretion by the ant's Malpighian tubules

The crude 15% TFA abdomen extracts had a toxic effect in the fluid secretion assay.
In the presence of the honeybee's extract the fluid secretion ceased within the first 10 minutes of the test period (Fig. 4.1A). There was no clear recovery on wash-out, only in one of four experiments the recovery was clear and consistent. The ant's extract (Fig. 4.1B) provoked a transient stimulation to 198% ± 32 ($p \leq 0.05$, n=5) which was followed by an irreversible inhibition

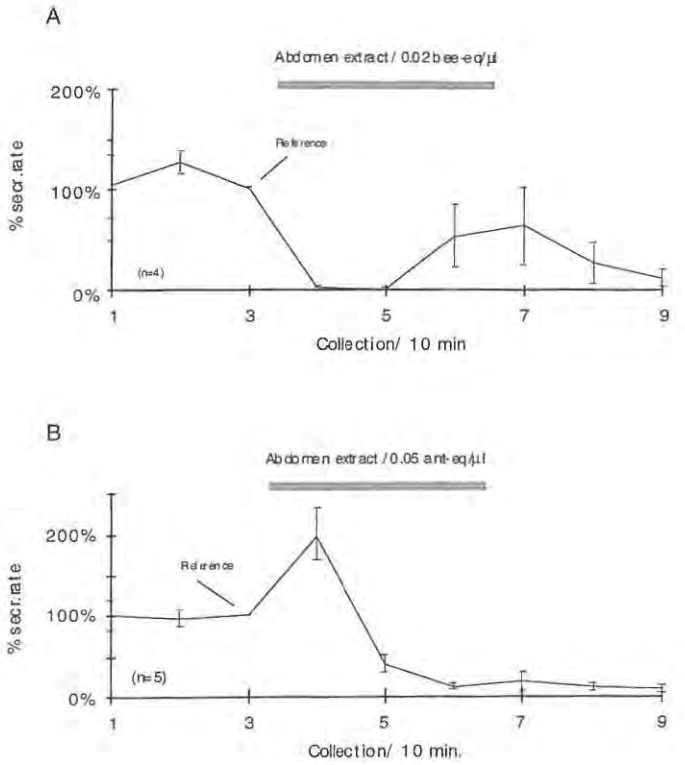

Figure 4.1. Effect of 15% TFA abdomen extracts on the fluid secretion rate of single isolated Malpighian tubules of the forest ant, *F. polyctena*. **A**. The effect of the honeybee's extract, tested at a concentration of 0.02 bee equivalents (bee-eq)/µl. **B.** The effect of the ant extract, tested at a concentration of 0.05 ant equivalents (ant-eq)/µl. The presence of the extract is indicated by the bars. Mean values ± S.E.M., (n=number of tubules tested).

Effects of the different prepurified fractions of the bee's abdomen extract in fluid secretion experiments

Prepurification of the bee's abdomen extract over the C_{18} cartridges resulted in three separate CH_3CN fractions (20, 40 and 65%), characterized by a stepwise increase in hydrophobicity of the eluted material. They were tested in the fluid secretion assay (Table 1.). After fractionation the toxicity was confined to the 20% prepurified fraction: the fluid secretion rate fell within the first 10 minutes and was not restored upon washout of the test solution.

Table 1. *Effect of 0.02 bee-eq/µl of the different prepurified fractions of an abdomen extract on the fluid secretion rate of single isolated Malpighian tubules of the ant.*

	% secretion rate			
Fraction	Control	Test	Recovery	n
20% CH3CN	120 ± 8	7 ± 6 *	15 ± 11 *	4
40% CH3CN	115 ± 7	152 ± 42	113 ± 20	4
60% CH3CN	100 ± 3	58 ± 3 *	64 ± 10	4

The third collection was taken as 100%. * Significantly different from the controls (unpaired Student *t*-test, $p \leq 0.05$). Mean values ± SE, n = number of tubules tested.

A transient stimulation was observed with the 40% prepurified fraction. After a twofold increase, seen during the first collection of the test period, the primary urine production returned to the control value within the next 10 min collection period, which explains the value of 152% in table 1. The 60% prepurified fraction on the other hand caused an inhibition which was maintained throughout the test period and which was reversible upon washout. The results suggested that the abdominal extract contained both diuretic and antidiuretic factors. FopADF was purified previously from the 60% prepurified fraction of a crude 15% TFA abdominal extract of the ant (Laenen et al., 1998a). As the 60% prepurified fraction of the bee's crude abdomen extract, showed a reversible antidiuretic effect on fluid secretion, it was decided to use this fraction for further purification and comparison.

Chromatography of the 20% prepurified fraction of the 15% TFA bee's abdomen extract, compared with melittin.

In an effort to explain the apparant toxic effect of the 20% prepurified fraction, three bee equivalents of this fraction were separated on an analytical C18 column and the resulting

chromatogram was compared to the elution of melittin (Fig. 4.2), the main component of bee venom (Habermann, 1972) under identical conditions.

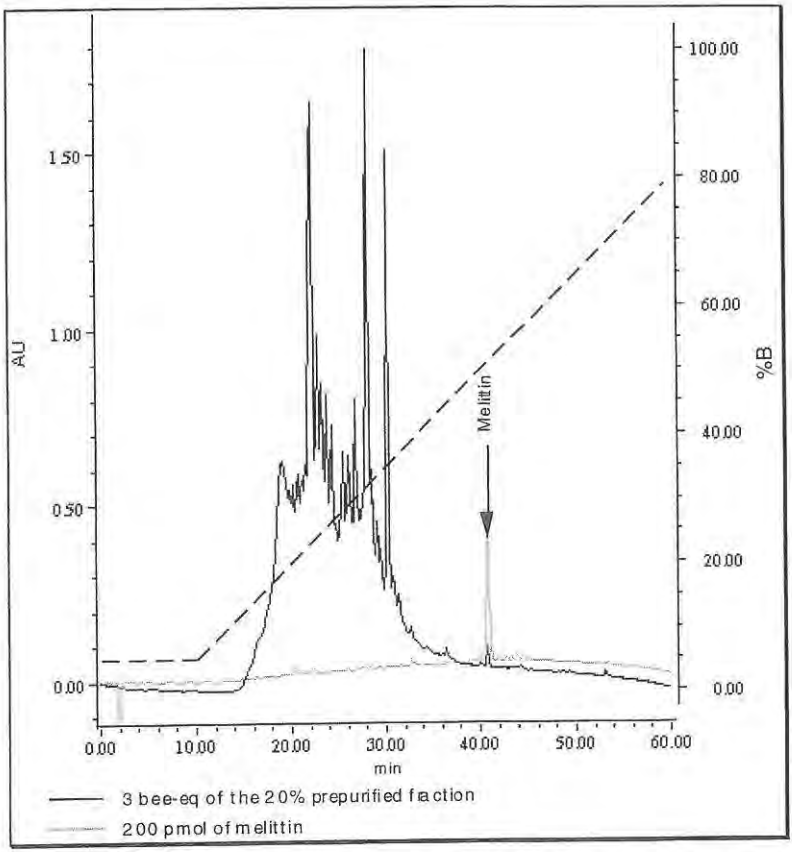

Figure 4.2. U.V. absorbance profile of three bee-eq of the 20% prepurified fractions of the 15% TFA abdomen extract, separated on the Waters Delta-Pak HPI C18 (5µm, 300Å, 3.9x150 mm) column. The elution time of melittin is shown (arrow). Conditions are described in Materials and Methods. The dashed line represents the concentration of solvent B.

Chromatography of the 60% prepurified fraction of the 15% TFA bee's and ant abdomen extract

Approximately 50 bee-eq of the 60% prepurified fraction were lyophilised and redissolved in 20 ml of 5% CH_3CN, 0.1% TFA. This solution was loaded onto the analytical C_{18} column by means of one of the solvent delivery pumps. The same elution conditions as for the ant's extract (Laenen et al., 1998a) were used.

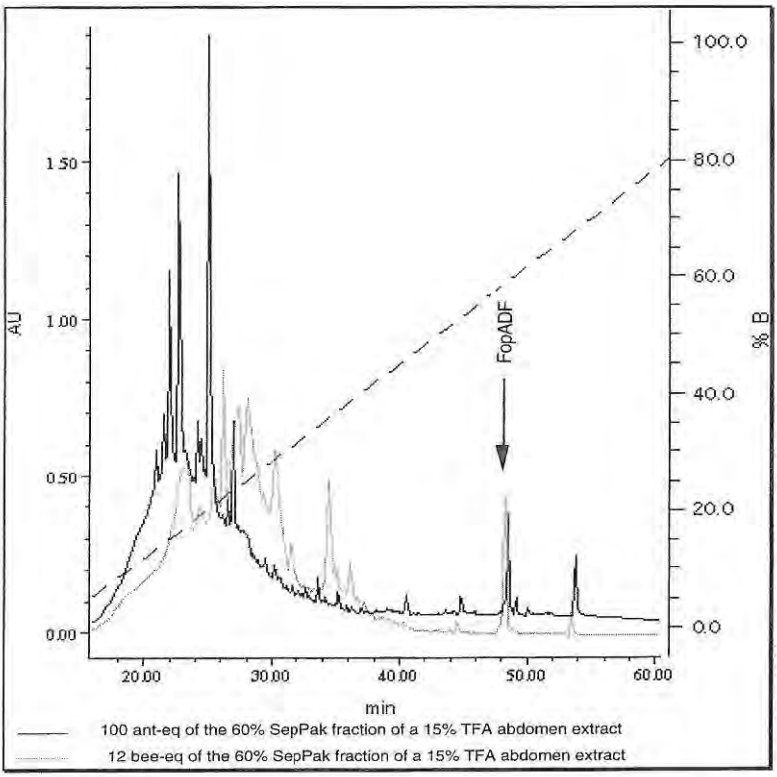

Figure 4.3. U.V. absorbance profile of the 60% prepurified fractions of 15% TFA abdomen extracts of the ant and the honeybee, separated on the Waters Delta-Pak HPI C18 (5µm, 300Å, 3.9x150 mm) column. The elution time of FopADF is shown (arrow). Conditions are described in Materials and Methods. The dashed line represents the concentration of solvent B.

Compared to the chromatogram for 100 ant-equivalents (ant-eq), a shift indicating the presence of more hydrophobic material could be observed (Fig. 4.3). The main U.V.-absorbing material eluted later and the elution is spread over a longer time interval. The chromatogram was less resolved and clearly different from that of the ant's extract.
Since we tried to identify an antidiuretic factor in the bee's abdomen extract, the elution of the chromatographic peak fractions was compared to the elution of the *F. polyctena* antidiuretic factor (FopADF).

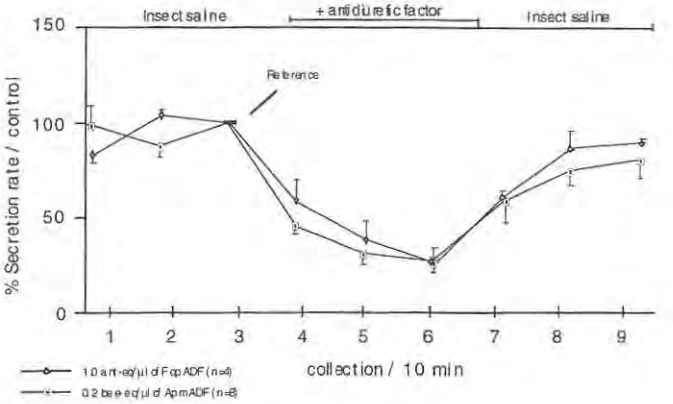

Figure 4.4. Effect of the antidiuretic factors on the fluid secretion rate of single isolated Malpighian tubules of the forest ant, *F. polyctena*. FopADF is *F. polyctena* antidiuretic factor; ApmADF is *A. mellifera* antidiuretic factor. The presence of the factors is indicated by the bars. Mean values ± S.E.M., (n=number of tubules tested).

One fraction eluted at 48.3 minutes which was almost identical to the elution of FopADF. Two bee-eq of this fraction were lyophilised, redissolved in 100 µl insect saline and tested in the fluid secretion assay on single isolated Malpighian tubules of the forest ant. A test concentration of 0.02 bee-eq/µl inhibited the fluid secretion in a reversible way to 32% ± 5 ($p \leq 0.05$, n=8). Recovery was 86% ± 10 on washout. The factor was further referred to as *A. mellifera* antidiuretic factor (Apm-ADF). The response to Apm-ADF was not statistically different ($p=0.708$, unpaired Student's *t*-test) from that obtained with the ant's antidiuretic factor (Fig. 4.4). Tested at a concentration of 0.5 ant-eq/µl, FopADF reversibly reduced the primary urine production to 37% ± 7 ($p \leq 0.05$, n=4), recovery to

72% ± 19 on washout. The effect of FopADF isolated from the batch of ants used in the present study confirms our previous findings (Laenen et al., 1998a).

Effect of FopADF and ApmADF on the membrane potentials of single isolated Malpighian tubules of the forest ant

The apparent similarity between FopADF and ApmADF was further investigated by measuring the effect of both factors on the membrane potentials of single isolated Malpighian tubules of the ant (Fig. 4.5).

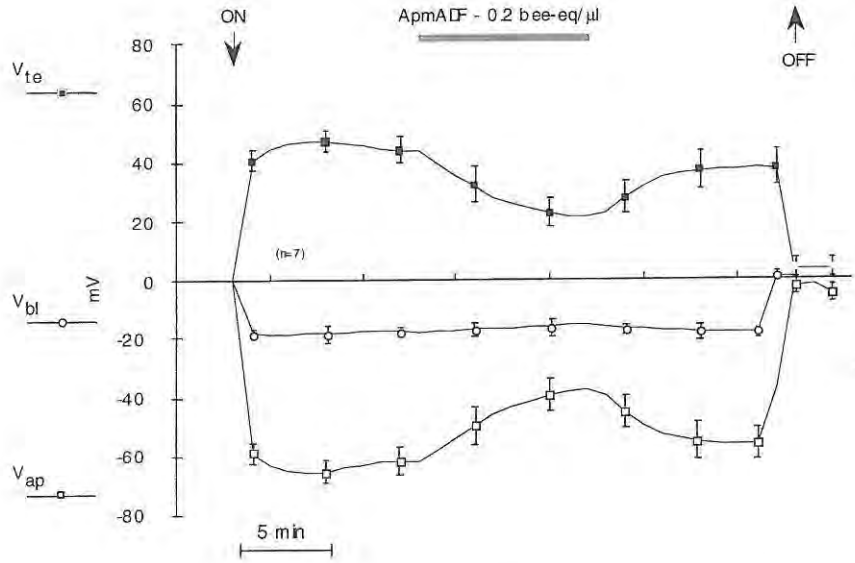

Figure 4.5 Running mean for the effect of *A. mellifera* antidiuretic factor (ApmADF), tested at a concentration of 0.2 bee-eq/µl on the transepithelial (V_{te}) and the basolateral (V_{bl}) membrane potential measured simultaneously. Presence of the factor is indicated by the bar. Arrows indicate impalement (ON) or withdrawal (OFF) of the microelectrodes. Mean values ± S.E.M., (n=number of tubules tested).

ApmADF was applied at a test concentration of 0.2 bee-eq/µl and elicited a significant depolarisation of the transepithelial potential by 22.8mV ± 1.5 ($p<0.01$,n=6). This effect recovered to 83% ± 7 (n=6) of the control value. A similar response was obtained

previously for FopADF (Laenen et al., 1998a). Statistical analysis with an unpaired Student's *t*-test did not show any significant difference ($p = 0.401$) between the effects of ApmADF and FopADF.

Less resemblance existed for the effect of both factors on the basolateral membrane potential. At a test concentration of 0.2 bee-eq/µl Apm ADF elicited a small depolarisation of the basolateral membrane potential by 2.1mV ± 0.9 ($p<0.01$, $n=7$). This small depolarisation is in contrast with the large depolarisation of V_{bl} observed when 1.0 ant-eq/µl of FopADF was applied (Laenen et al., 1998a). The difference in response is significant ($p \leq 0.05$, unpaired Student's *t*-test). In both cases the reponse was reversible.

Are diuretic factors present in the 10% TFA bee's head extract ?

We also tried to find out whether a *F. polyctena* diuretic peptide (Fop-DP) analogue was present in the 10% TFA head extract of the honeybee *A. mellifera*. Fop-DP was freshly purified as described previously (Laenen et al., 1998b) from the 20% prepurified fraction of a 10% TFA head / thorax extract of 15,000 ants. It eluted at 24 min from the Waters Delta-Pak HPI C18 (5µm, 300Å, 3.9x150 mm) column.

From the 10% TFA bee's head extract, 12 bee-eq of the 20% prepurified fraction were concentrated by means of centrifugal evaporation, redissolved in 5% CH_3CN, 0.1% TFA and loaded on to the analytical C_{18} column. Elution conditions were the same as for the ant's extract, but resulted in a much broader chromatogram (Fig. 4.6). The onset of U.V.-absorbing material is the same as for the ant's extract but the elution is spread over a longer time interval. Since we tried to identify a diuretic factor in the bee's head extract, the elution of the chromatographic peak fractions was compared to the elution of Fop-DP under identical conditions.

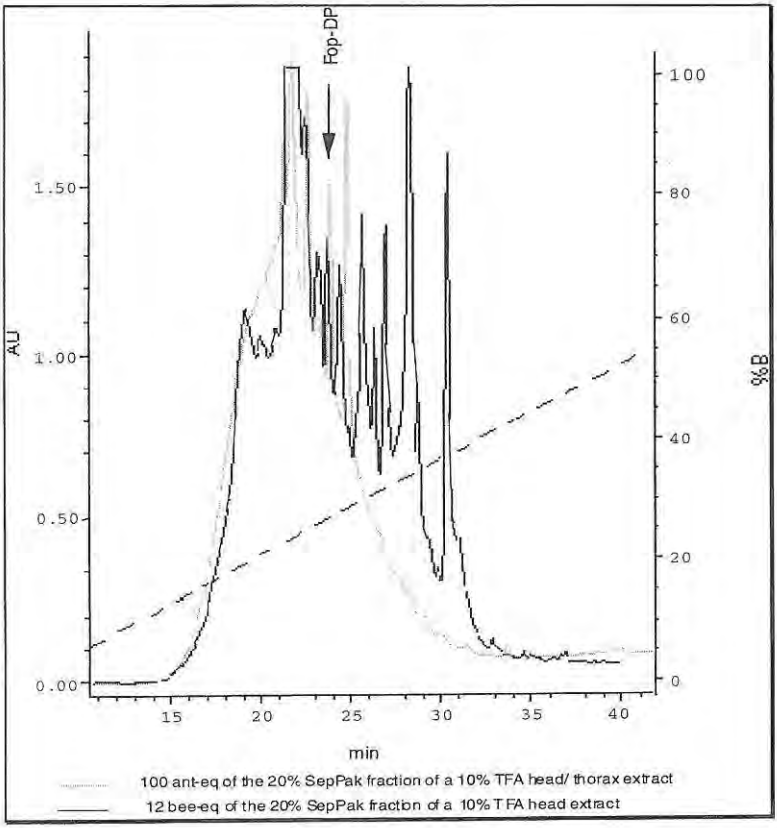

Figure 4.6. U.V. absorbance profile of the 20% prepurified fractions of 10% TFA head extracts of the ant and the honeybee, separated on the Waters Delta-Pak HPI C18 (5µm, 300Å, 3.9x150 mm) column. The elution time of Fop-DP is shown (arrow). Conditions are described in Materials and Methods. The dashed line represents the concentration of solvent B.

One fraction eluted at 23.8 minutes which is almost identical to the elution for Fop-DP (Laenen et al., 1998b). Two bee-eq of this fraction were lyophilised, redissolved in 100 µl insect saline and tested in the fluid secretion assay on single isolated Malpighian tubules of the forest ant. The active material in this fraction, further referred to as *A. mellifera* diuretic factor (Apm-DF), induced a small diuretic response of 31% ± 12 ($p \leq 0.05$, n=4), which recovered to 80% ± 7 on washout (Fig. 4.7). The effect was much smaller ($p \leq 0.05$,

unpaired Student's *t*-test) than the effect obtained with the ant's diuretic peptide (Laenen et al., 1998b). Tested at a concentration of 0.01 ant-eq/µl, Fop-DP reversibly tripled the primary urine production.

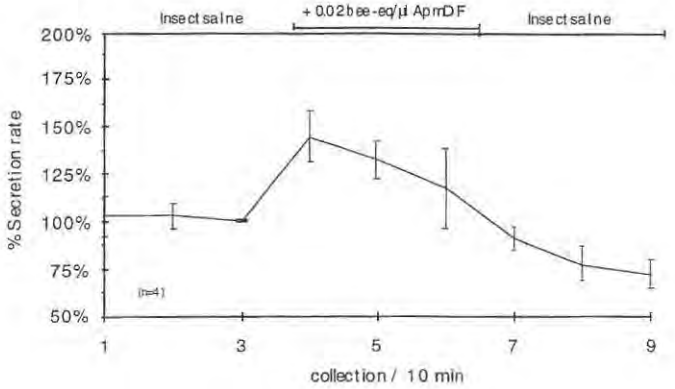

Figure 4.7. Effect of *A. mellifera* diuretic factor (Apm-DF), tested at a concentration of 0.02 bee-eq/µl on fluid secretion by isolated Malpighian tubules of the forest ant *F. polyctena*. Presence of the factor is indicated by the bar. Mean values ± S.E.M., (n=number of tubules tested).

DISCUSSION

Comparison of the effect of the crude abdomen extracts on fluid secretion in the Malpighian tubules of the ant

For both insect species the abdomen extract had an irreversible inhibitory effect on the primary urine production of the ant's Malpighian tubules. In the ant's extract the stimulation seen during the first collection period, is suppressed and followed by a reduction of the fluid secretion rate. This suggested that the extract contained both diuretic and antidiuretic factors and confirmed the results of a previous study on an ant's abdomen extract (Laenen et al., 1998a). Diuretic factors were shown to be present in the 20% prepurified fraction of this extract and an antidiuretic factor could be identified in the 60% prepurified fraction (see Chapter 1). A small difference with the present study was that the batch of animals used this time seemed to contain relatively more diuretic material than the

batch of animals used in the previous study. In that study the crude extract did not provoke a transient stimulation, preceding the inhibition.

The bee's 15% TFA abdomen extract seemed to have a toxic effect on the single isolated Malpighian tubules of the ant. The presence of bee venom components in the crude extract may be responsible for this toxicity. The venom sac has an average weight of 0.3 mg (Crane, 1990) which is approximately 1% of the abdominal weight. Due to the extraction procedure used, venom components were present in the crude extract. The main component of bee venom with respect to weight (it is 50 percent of dry venom) and activity is the polypeptide melittin (Habermann, 1972). It is a bioactive peptide with strong surfactant activity. This physicochemical activity of melittin causes an increase in cell permeability which results in cell lysis. The toxicity of melittin is enhanced in the presence of phospholipase A, the second component of the venom with respect to weight . Phospholipase A is the most important antigen of the venom and hydrolyses the membrane phospholipids of cells (Kemeny et al., 1983). The physiological activity of diuretic factors in the crude extract may have been obscured by the presence of these toxic components. Fractionation of the crude extracts in prepurified fractions as performed in the present study (see below) helped to uncover the factors with a physiological and direct effect on the primary urine production in single isolated Malpighian tubules of the ant.

Interpretation of the response obtained with the different prepurified fractions of the bee's abdomen extract

Prepurification of the 15% TFA abdomen extract of the honeybee *A. mellifera* over C_{18} cartridges resulted in three CH_3CN fractions characterized by a stepwise increase in hydrophobicity. The toxicity of the crude extract was recovered in the 20% fraction and could be explained by the presence of bee venom components, i.e. melittin. Melittin is a lytic peptide and the major component of bee venom (Habermann, 1972). Since it elutes within the retention times of U.V.-absorbing material, present in the 20% prepurified fraction (Fig. 4.2), it is probably present in this fraction and much less or not at all in the 40% prepurified fraction.

The 40% fraction had a significant diuretic effect, suggesting the presence of at least one diuretic factor. Diuretic factors were shown to exist in the honeybee. Corpora allata extracts of the honeybee stimulated the amaranth clearance of isolated Malpighian tubules of the bee by 30% (Altmann, 1956) and increased the tubule writhing frequency. This combination of effects, stimulation of fluid secretion rate and stimulation of tubule writhing frequency, has also been observed for the corticotropin releasing factor (CRF) - like

peptides in other insects (Coast, 1998). The CRF-like peptides, range in size from 30 to 46 amino acids, and have an average sequence homology of 50%. Their mode of action appears to be relatively uniform across insect species and is mediated through an increase in intracellular cAMP (Audsley et al., 1995). Moreover, up till know all members of the CRF-like diuretic peptide family were recovered in the 40% fraction after prepurification of the crude extracts over Waters C_{18} or C_4 cartridges (Clottens et al., 1994),(Furuya et al., 1998). So, it might be that one of the diuretic factors present in the bee's abdomen extract is related to the CRF-like family of diuretic peptides.

The 60% prepurified fraction of a 15% TFA abdomen extract of the honeybee had an antidiuretic effect on the fluid secretion in the Malpighian tubules of the forest ant. An antidiuretic effect on the Malpighian tubules of the bee had previously been found in corpora cardiaca extracts also (Altmann, 1956). The corpora cardiaca are the classical neurohemal organs in insects in which neurohormones produced in neurosecretory cells of the brain are stored and released into the hemolymph (Orchard, 1983). This suggests the presence of antidiuretic factors in the most important neurosecretory organ and helps to explain the antidiuretic response for the abdomen extract, containing the hemolymph in contact with the Malpighian tubules. A similar antidiuretic effect has been observed for the 60% CH_3CN fraction of a 15% TFA abdomen extract of the ant and resulted in the chromatographic purification of FopADF (Laenen et al., 1998a). In the chromatographic analysis of the bee's extract a factor was identified which coeluted with FopADF and which had similar effects in ant tubules.

Biological activity of the antidiuretic factor of the honeybee in ant tubules: a model.

The antidiuretic response of the 60% prepurified fraction seemed to be due to the presence of a factor with the same retention time as FopADF. This factor, ApmADF, inhibited the primary urine production of the ant's Malpighian tubules in an identical way as FopADF ($p=0.708$, unpaired Student's *t*-test). The similarity was only partial when the effect of ApmADF on the membrane potentials was measured. In contrast to FopADF, ApmADF elicited only a small, but significant depolarisation of the basolateral membrane potential, but, like FopADF, it had a major effect on the transepithelial potential. Since ApmADF had only a small effect on the basolateral membrane potential, the change in transepithelial potential primarily reflected the depolarisation of the apical membrane potential ($V_{ap} = V_{bl} - V_{te}$).

Taken together with the result in the fluid secretion assay, the inhibition of transport by ApmADF seems to be due primarily to an effect on the apical membrane. Since the

electrogenic V-type H^+-ATPase is located in this membrane (Zhang et al., 1994) it is a possible target for both FopADF and ApmADF.

Based on previous studies a model for KCl transport in *F. polyctena* Malpighian tubules in control conditions was proposed (Van Kerkhove, 1994). In this model potassium is transported against both a concentration and an electrical gradient (Leyssens et al., 1992). The active translocation of potassium from hemolymph to lumen is parallelled by passive transport of Cl^- and water across the epithelium (Dijkstra et al., 1994).

The transcellular transport of potassium consists of K^+ uptake at the basolateral membrane and K^+/H^+ antiport at the apical membrane. A V-type H^+ ATPase in the apical membrane creates an electrochemical H^+-gradient directed from lumen to cell (Zhang et al., 1994). This electrochemical gradient can be used by the antiporter to extrude K^+ from the cell into the lumen, in exchange for protons. The basolateral uptake of potassium occurs primarily through channels, at least in the presence of a high K^+ concentration. At lower hemolymph potassium concentrations electroneutral K^+/Cl^- and $Na^+/K^+/2Cl^-$ cotransporters become important (Leyssens et al., 1994).

According to the model ApmADF could affect either K^+ uptake, K^+ extrusion or both. The V-type H^+ ATPase is a possible candidate for regulation of salt and fluid secretion in the Malpighian tubules of the ant. The finding that ApmADF reduced V_{ap} indicates that the proton pump may indeed be a target for the antidiuretic factor. Similar effects (depolarization of V_{ap}) were found for different drugs with an inhibitory effect on the apical H^+-pump. Two typical V-type H^+ ATPase inhibitors, N-ethylmaleimide (NEM) and bafilomycin-A1 (Baf-A1) depolarised the apical membrane potential and reduced the fluid secretion rate (Casteels et al., 1990) in single isolated Malpighian tubules. Like ApmADF none of these substances had an effect on the basolateral membrane potential.

Chromatographic comparison of the 20% prepurified honeybee's and forest ant's head / thorax extracts

The 20% prepurified fractions of both 10% TFA head / thorax extracts were chromatographically compared. In the resulting chromatograms a clear shift indicating the presence of more hydrophobic material was observed for the bee's extract. As for the abdomen extract, more material was present in the bee's extract, but since the protein concentration was not determined for these extracts, it can not be excluded that simply more bee's extract was loaded onto the analytical C_{18}-column. A diuretic factor with the same elution time as Fop-DP could be identified in the bee's head / thorax extract. As

mentioned above, diuretic factors were shown to exist in the honeybee. Corpora allata extracts of the honeybee stimulated the amaranth clearance of isolated Malpighian tubules of the bee, but by only 30% (Altmann, 1956) and increased the tubule writhing frequency. Apm-DF did have a stimulatory effect on tubules from the ant, although the effect was small. Possibly the ant tubules are not very sensitive to Apm-DF. Whether Apm-DF acts as an endogenous diuretic peptide in the honeybee can only be investigated in fluid secretion experiments on single isolated Malpighian tubules of the honeybee.

CHAPTER 5

Immunological and chromatographic evidence for the presence of a CRF-related diuretic peptide in head / thorax extracts of the forest ant, *Formica polyctena.*

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Running head: CRF-related factor(s) in head / thorax extracts of the ant

ABSTRACT

A 15% trifluoroacetic acid (TFA) head / thorax extract of the forest ant *Formica polyctena* was prepurified on C_4 reversed-phase cartridges eluted with increasing concentrations of CH_3CN. An immunological investigation with polyclonal antisera raised against two insect corticotropin-releasing factor related diuretic hormones, *Locusta*-DH, Lom-DH and *Manduca*-DH, Mas-DH showed the presence of immunoreactive material in the 40% CH_3CN fraction.

Head/thorax extract prepared in acidic methanol or in 15% TFA was prepurified on C_4 cartridges and the 40% CH_3CN eluant was fractionated by reversed-phase HPLC on an analytical C_8-column. Fractions were tested for their ability to stimulate fluid secretion and cAMP production by ant Malpighian tubules. Biological activity was restricted to one or two fractions which had a retention time similar to that of Lom-DH when chromatographed under identical conditions.

These results strongly suggested that a corticotropin-releasing factor-related diuretic hormone was present in *Formica*.

Keywords : insect, HPLC, Malpighian tubule, cAMP, enzyme linked immunosorbent assay

INTRODUCTION

In insects two classes of peptides with a direct stimulatory effect on fluid secretion by isolated Malpighian tubules have been characterised. One class has structural homology with the vertebrate CRF (corticotropin-releasing factor) / urotensin / urocortin/sauvagine family of peptides and acts via an increase in intracellular cAMP (Coast et al., 1994). The first of these CRF-related diuretic peptides to be identified was purified from an acidic methanol extract of trimmed heads from tobacco hornworm (*Manduca sexta*) larvae (Kataoka et al., 1989). The peptide was shown to stimulate post-eclosion diuresis in head-ligated newly emerged adult butterflies (*Pieris rapae*) and was named *Manduca sexta* diuretic hormone (Mas-DH). Recently the receptors for Mas-DH and for a related peptide from *Acheta domesticus* (*Acheta*-DP; Acd-DP) have been cloned from a Malpighian tubule cDNA library and functionally expressed in COS-7 cells (Reagan, 1995),(Reagan, 1996). They belong to the calcitonin / secretin / CRF receptor family of G-protein coupled receptors all of which are positively coupled to adenylyl cyclase.

The identification of a second family of diuretic peptides (the insect kinins) was originally based on their ability to stimulate the spontaneous contractile activity of cockroach hindgut, but they were subsequently shown to accelerate fluid secretion by isolated Malpighian tubules. In this group of myotropic peptides, the myokinins, the C-terminal pentapeptide Phe-Xaa-Xaa-Trp-Gly-NH_2 is well conserved and is essential for both their myotropic and diuretic activity (Holman et al., 1990). In contrast to the CRF-related peptides, these peptides do not act via cAMP (Coast, 1996),(O'Donnell et al., 1996) but use calcium as an intracellular second messenger. Kinins and CRF-related diuretic peptides have been shown to act synergistically in controlling fluid secretion by *Locusta* Malpighian tubules (Coast, 1995): at low concentrations, they act co-operatively to produce a diuretic response that is greater than the sum of their separate activities. This appears to be of physiological relevance, because the increase in circulating level of Lom-DH following a meal is not sufficient to stimulate diuresis maximally, but will do so in the presence of a very low concentration of *Locusta* kinin. The synergistic effect of two stimulants acting via different second messengers resembles that between biogenic amines (octopamine and 5-hydroxytryptamine, 5-HT) and cardioacceleratory peptides in the excitation of the heart of adult *M. sexta* (Prier et al., 1994).

In a previous study, the cockroach hindgut was used as a bioassay in a search for myotropic activity in ant head extracts (De Decker, 1993). This resulted in the purification of two tachykinins, Fop-TK I and Fop-TK II, but did not yield any myokinins although

other uncharacterised myotropic activities were present. In the present study, we have shown that extracts of ant heads/thoraces contain a factor that stimulates fluid secretion and cAMP production in ant Malpighian tubules. This material is recognised by antisera raised against *Locusta*-DH (Lom-DH) and Mas-DH, and has a similar retention time to Lom-DH on reversed-phase HPLC, consistent with it being a CRF-related diuretic peptide.

MATERIAL AND METHODS

Insects

The ants used in this study were collected from their natural nest in the forest of Slederlo (Genk, Belgium). They were reared at 25°C and 65% relative humidity under a 16h:8h light:dark cycle, and fed a diet of sugar and water.

Experimental solutions

The composition of the insect saline was based on the composition of the ant's hemolymph (Van Kerkhove et al., 1989) and contained (in mmol/l) : 27.0 KCl, 8.04 K_3citrate, 1.97 $CaCl_2$, 13.0 $MgCl_2$, 16.8 Na_2fumarate, 14.4 Na_2succinate, 2.80 L-alanine, 10.5 trehalose, 11.7 maltose, 138.8 glucose and 12.1 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (Hepes). The saline had an osmolality of 350 mosm/kg and was adjusted to a pH of 7.2 by means of NaOH. Synthetic Lom-DH and Mas-DH were prepared in Birkbeck College, London. Polyclonal antisera to Lom-DH and to Mas-DH were a kind gift from the laboratory of Dr. L. Schoofs (Zoölogisch Instituut, Leuven).

Preparation of an acidic methanol head/thorax extract.

Approximately 50,000 ants were collected from the natural nest in the forest of Slederlo (Genk, Belgium) and instantly frozen in liquid nitrogen. Subsequently the frozen body parts, head, thorax and abdomen, were separated from one another by sieving and stored in liquid nitrogen until further use. One hundred grams of heads/thoraces (about 20,000 ant-equivalents (ant-eq)) were divided between two 250 ml centrifugation bottles kept on ice. A volume of 150 ml of acidic methanol (87% methanol, 5% acetic acid, 8% distilled water) was added, and the heads / thoraces were homogenised with a Heidolph (Vac 600) mixer at 13,000 rpm using two 2 minute bursts. This extract was kept on ice for 10 min prior to being centrifuged in a cooled (4°C) Beckman JA14 rotor at 14,000 rpm for 20 minutes. The supernatant was filtered through Whatmann N°1 filter paper and temporarily

stored at 4°C. The pellet was resuspended in 150 ml of acidic methanol and extracted for a second time as described above. The supernatants were pooled and distilled water was added to dilute the methanol to a final concentration of 50%. This material was then frozen in liquid nitrogen and connected to a HetoVac II lyophilisator in order to evaporate the methanol. The methanol-free solution was transferred to a 250 ml centrifugation bottle and an equal volume of ethyl acetate was added. This mixture was shaken vigorously and left on ice for 10 min prior to centrifugation for 30 min (13,000 rpm, 4°C) in a Beckman centrifuge (rotor type JA14). The ethyl acetate, the upper liquid phase, was replaced by hexane, and the bottle was shaken and stored overnight at -70 °C. By the following morning the supernatant was frozen while the hexane was still liquid and could easily be decanted. After the hexane was removed, the supernatant was thawed and filtered over Whatmann N°1 filter paper prior to prepurification using custom-made 4cc Vydac C_4 syringe cartridges (Varian). The cartridges had previously been activated with acetonitrile (CH_3CN) and were equilibrated in milliQ (Millipore) water, both solutions contained 0,1% TFA. The cartridges were eluted stepwise with 90 ml of 20%, 40% and 65% CH_3CN in constant 0,1% TFA. These prepurified fractions were lyophilised and stored in the freezer at -70°C until further use.

Preparation of a 15% TFA head / thorax extract

A head / thorax fraction of 50 g (± 10,000 ant-eq) was divided between four 30 ml centrifugation tubes kept on ice. To each tube 25 ml of ice-cold 10% trifluoroacetic acid (TFA) was added and their contents were homogenised with a Heidolph mixer at 13,000 rpm using two 2 minute bursts. The extract was left on ice for 10 min prior to centrifugation in a cooled (4°C) Beckman JA14 rotor at 14,000 rpm for 20 minutes. The supernatants were collected and filtered in sequence through Whatmann N°42 and Whatmann N°1 filter paper prior to prepurification using custom-made 4cc Vydac C_4 syringe cartridges (Varian) as for the methanolic extract described above.

Chromatographic fractionation of the crude extracts

The prepurified material from both the methanolic and the 15% TFA extract were chromatographically compared by reversed-phase HPLC on Waters equipment consisting of two 510 pumps, a 486 tuneable absorbance detector set at 214 nm and the Millennium 2.15 chromatography manager. For both extracts, 2,500 ant-eq of the 40% CH_3CN prepurified material were lyophilised and subsequently redissolved in 10 ml of solvent A

(5% CH_3CN, 5 mM TFA). This solution was loaded onto a Waters Nova-Pak C_8 (4.6 x 250 mm, 60 Å, 4 µm) column and eluted using the following conditions: a linear gradient of 5 - 30% of solvent B (60% CH_3CN, 5 mM TFA) over 5 min followed by a linear gradient of 30 - 70 % of solvent B over 60 min; flow rate 0.63 ml/min. Fractions were collected at two minute intervals using a Waters 5.302 fraction collector. Samples of these fractions, each containing 1000 ant-eq, were dried in polypropylene eppendorf tubes by centrifugal evaporation. They were redissolved in insect saline and used to monitor for biological activity, namely for effects on fluid secretion and cAMP production by isolated Malpighian tubules.

ELISA for CRF-like material

The immunological detection of CRF-like material in the 15% TFA head / thorax extract was carried out as described by Patel *et al.* (1994) (Patel et al., 1994) using rabbit polyclonal antisera to Mas-DH and Lom-DH. Samples were prepared as follows: aliquots (n=3) of the 40% CH_3CN eluant from the C_4 cartridge containing the appropriate amount of ant-eq (specified in the Results section) were dried by centrifugal evaporation, resuspended in 4 µl 60% acetonitrile and then diluted to 100 µl with coating buffer (0.1 M carbonate/bicarbonate, pH 9.6). A 4 µl sample taken from the 60% CH_3CN eluant and diluted to 100 µl with coating buffer served as a negative control. As a positive control and internal reference, a 1 pmol sample of Mas-DH was used. Samples were added to the wells of a microtitre plate and incubated overnight at 4°C. After this incubation, the plate was washed twice with 10 mM phosphate buffer / 0.1% Tween-20 (PB). Blocking solution (2% non-fat milk in PB) was then added to each well and the plate was incubated at 37°C for 90 min. The plate was washed again with PB (twice), and then 100 µl of primary antiserum (1:3000 dilution in PB) was added. The plate was incubated for a further 90 min at 37°C and, after another PB wash, 100 µl of goat anti-rabbit antiserum (1:3000 in PB) conjugated with horseradish peroxidase was added to each well. The plate was incubated for another 40 min at 37°C and, after a final PB wash, 100 µl of substrate solution (4.25 mg *p*-nitrophenyl phosphate and 1.25 µl 30% hydrogen peroxide in 25 µl 0.1M citrate buffer, pH 5.0) was added to each well. At the end of a 40 min incubation at 37°C, 50 µl of 0.5M sulphuric acid was added to the wells and the absorbance was read at 410 nm on a Labsystems Multiscan MCC / 340.

Determination of cAMP concentration

The intracellular concentration of cAMP concentration in ant Malpighian tubules was determined as described previously (Laenen *et al.*, 1998b) (Aston and White, 1974). Due to the low concentrations of cAMP present, a highly sensitive commercial competitive binding assay was used (^{125}I cAMP scintillation proximity assay kit, Amersham International, RPA538). In this assay each test tube contained approximately 45 tubules. Phosphodiesterase activity was inhibited by adding IBMX to each of the test tubes (final concentration of 10^{-4}M). After an incubation of 30 min, insect saline with or without test sample was added to the tubes which were incubated for another 30 minutes. Addition of 900 µl of ice-cold methanol and transfer to ice for 15 min stopped the reaction and precipitated the tissue proteins. The protein content of Malpighian tubules was determined using a BCA-protein determination kit (Pierce - N°23223) with bovine serum albumin (BSA) as standard.

Fluid secretion assay for a single isolated Malpighian tubule

To measure fluid secretion by isolated Malpighian tubules of the ant, we used the method described by De Decker *et al.* (1994) (De Decker et al., 1994). Briefly, each experiment consisted of measuring fluid secretion over three periods, with 3 measurements at 10 minute intervals during each period. The first period served as a control, and the rate of secretion during the third 10 minute collection was taken as a reference value. At the start of the next period, a test period, the bathing droplet was removed and replaced with 50µl of test solution. The small amounts of test solution available made it impossible to maintain a continuous bath perfusion during this test period as in the original method. However, stopping perfusion of the bath did not on its own have any effect on fluid secretion as seen in a set of ten control experiments (result not shown). All fluid secretion rates were compared to the reference value, set to 100%. The effect of a test substance was considered when it differed significantly from the reference value. Finally during the washout period, the perfusion of the bath with insect saline was switched on again and the test solution washed out.

Statistics

Results are given as mean values ± standard error (S.E.M.), with the number of tubules in parentheses. Statistical significance was calculated using the paired Student's *t*-test, unless indicated otherwise. A difference was considered significant for $p \leq 0.05$.

RESULTS

Effect of the head / thorax extracts on primary urine production

Samples from both the methanolic and the 15% TFA head / thorax extracts were tested for diuretic activity on isolated Malpighian tubules (Fig. 5.1). The methanolic extract was tested at a concentration of 0.01 ant-eq/µl and stimulated the fluid secretion to a peak value of 178 ± 31% ($p \leq 0.05$, n=9) of the reference value. A similar response was obtained with the 15% TFA head / thorax extract when tested at 0.03 ant-eq/µl, with urine production increasing to 216 ± 36% ($p \leq 0.05$, n=4).

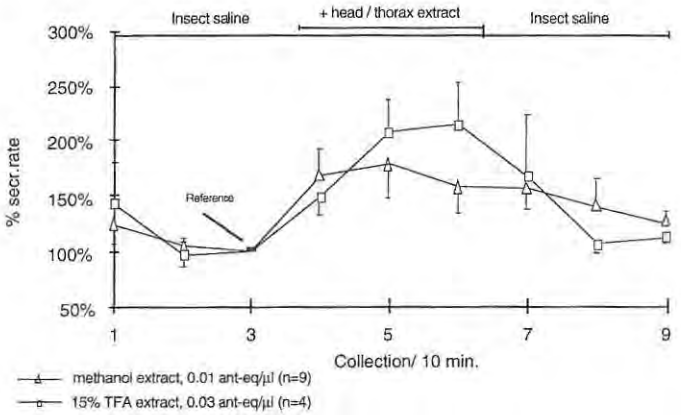


Figure 5.1. Effect of methanol or TFA head / thorax extracts on the fluid secretion rate of single isolated Malpighian tubules of the forest ant *Formica polyctena*. The presence of the extracts in the bath, is indicated by the bars. Mean values ± S.E.M. (n=number of tubules tested).

CRF-related diuretic peptides have been purified from material eluting in 40% CH_3CN from reversed-phase cartridges (Table 1).

Table 1 *Extraction procedure and elution behaviour of CRF-related peptides on analytical RP-columns*

Peptide	Extraction solvent	Active fraction	RP-column	Elution in % CH_3CN	Reference
Mas-DH	90% MetOH, 1% HOAc	35% CH_3CN	Aquapore RP300 C_8	33	(Blackburn *et al.*, 1991)
Manduca DP II	1M HOAc, 20 mM HCl	N.A.	Vydac C_4	29	(Kataoka *et al.*, 1989)
Lom-DH	90% MetOH, 1% HOAc	45% CH_3CN	Dynamax C_8	33	(Kay *et al.*, 1991)
Pea-DH	90% MetOH, 1% HOAc	45% CH_3CN	Dynamax C_8	37	(Kay *et al.*, 1992)
Acd-DH	90% MetOH, 1% HOAc	40% CH_3CN	Lichrosorb C_8	37	(Coast *et al.*, 1993)
Musca-DP	90% MetOH, 1% HOAc	40% CH_3CN	Deltabond C_8	36	(Clottens *et al.*, 1994)
Tem-DH	1M HOAc, 20 mM H_2SO_4	45% CH_3CN	Vydac C_4	30	(Furuya *et al.*, 1998)
	Spreading of CRF-related activity on analytical C_8-columns			33 - 37	

Reverse Phase column (RP-column), methanol (MetOH), acetic acid (HOAc), acetonitrile (CH_3CN).

This fraction from both extracts was therefore tested in the fluid secretion assay (Fig. 5.2). The response of Malpighian tubules to the methanolic extract was very variable. In each individual experiment, fluid secretion was increased when the 40% CH_3CN material was present in the bath, but the secretion varied from 184% to 607% for the last 10 minute collection during the test period, compared with the reference value.

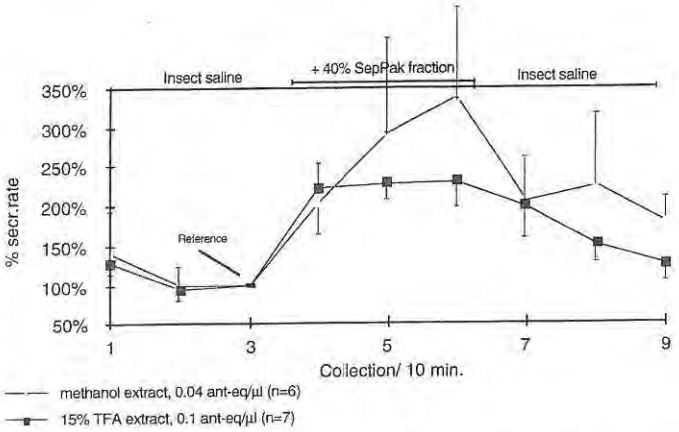


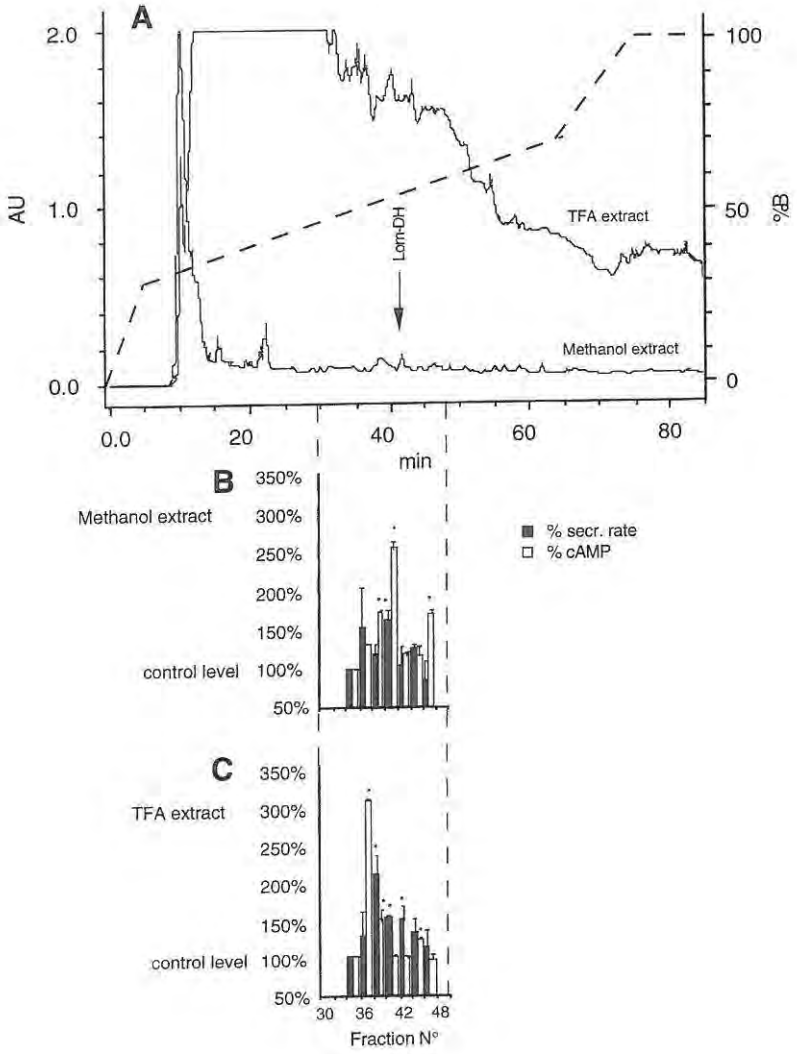
Figure 5.2. Effect of 40% CH_3CN prepurified fractions from methanol or TFA head/thorax extracts on the fluid secretion rate of single isolated Malpighian tubules of the forest ant *Formica polyctena*. The presence of the prepurified fractions in the bath, is indicated by the bars. Mean values ± S.E.M. (n=number of tubules tested).

For the test period as a whole (average of three 10 min collections), fluid secretion was stimulated to 273% ± 45 ($p \leq 0.05$, n=6) with 0.04 ant-eq/µl of the 40% CH_3CN material in the bath. This same fraction from the 15% TFA head / thorax extract also stimulated fluid secretion to 227% ± 27 ($p \leq 0.01$, n=7) at a concentration of 0.1 ant-eq/µl. However, although the overall effect of the TFA extract was similar to that of the methanolic extract, the variability in response was much less pronounced, as seen by the smaller error bars (Fig. 5.2).

Chromatographic comparison of the methanol and the TFA extract.

Material (2,500 ant-eq) from both the methanolic and the 15% TFA head / thorax extracts that eluted in 40% CH_3CN from the C_4 cartridges was analysed on an analytical C_8 column (Waters, NovaPak). For both extracts, U.V.-absorbing material eluted between 20% and 40% CH_3CN, but they clearly differed in the amount of material present; the 15% TFA extract contained at least twice as much material as the methanolic extract (Fig. 5.3A). To characterise the elution behaviour of CRF-related peptides on the NovaPak C_8 column, 20 pmol of synthetic Lom-DH was loaded onto the column. It eluted with a retention time of 40-41 min, equivalent to 32% CH_3CN, which was comparable to the retention time of other known CRF-related peptides (Table 1) on analytical reversed-phase columns. We therefore tested material from ant head / thorax extracts that eluted between 36 and 46 minutes for diuretic and cAMP generating activities (see below).

Figure 5.3. **A.** U.V. absorbance profile of 2,500 ant-eq of the 40% prepurified fractions from methanol and TFA head / thorax extracts, separated on a Waters Nova-Pak C_8 column (60Å, 4.6x250mm). The elution time of synthetic *Locusta migratoria* diuretic hormone (Lom-DH) is shown (arrow). Conditions are described in Materials and Methods. The dashed line represents the concentration of solvent B. **Panel B** shows the effects of the fractions obtained from the methanol extract on single isolated Malpighian tubules of the ant. For the cAMP assay a test concentration of 20 ant-eq/µl was used and the results were expressed as a percentage, the controls, in the presence of 10^{-4}M IBMX alone being set at 100% (mean values ± S.D., n = 2 sets of assays). In the fluid secretion assay the fractions were tested at 10 ant-eq / µl and results are expressed as the percentage of fluid secretion rate, compared to the internal reference (n=4).
Panel C shows the effects of the fractions obtained from the TFA extract on single isolated Malpighian tubules of the ant. The results are expressed as for the methanol extract and the assays were performed in the same way, using the same test concentrations.
* Significantly different from 100% ($p \leq 0.05$, paired Student's *t*-test).


A
2.0
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TFA extract
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min
B
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250%
200%
150%
control level 100%
50%
% secr. rate
% cAMP
C
TFA extract
350%
300%
250%
200%
150%
control level 100%
50%
30
36
42
48
Fraction N°

Diuretic activity of the chromatographic fractions in the fluid secretion assay

The lyophilised chromatographic fractions were redissolved in insect saline at a concentration of 10 ant-eq/µl 20 minutes prior to their use in the diuretic assay. For the methanolic extract (Fig. 5.3B), only the fraction eluting between 40 and 42 min (fraction 40) had a significant effect on fluid secretion, which was increased to 162 ± 16% ($p \leq 0.05$, n=4). In the TFA extract (Fig. 5.3C), diuretic activity was spread over two neighbouring fractions. The fraction eluting between 38 and 40 min stimulated fluid secretion to 209 ± 23% ($p \leq 0.01$, n=6), whereas the response to the next fraction eluting between 40 and 42 min was 151 ± 3% ($p \leq 0.05$, n=3). Lom-DH, a synthetic CRF-like peptide, was also tested for its effect on Malpighian tubules of the ant. Tested at a concentration of 2.5 µM, known to elicit a maximal diuretic response on *Locusta* tubules (Audsley et al., 1995), it had no effect on primary urine production by ant tubules (Fig. 5.4).

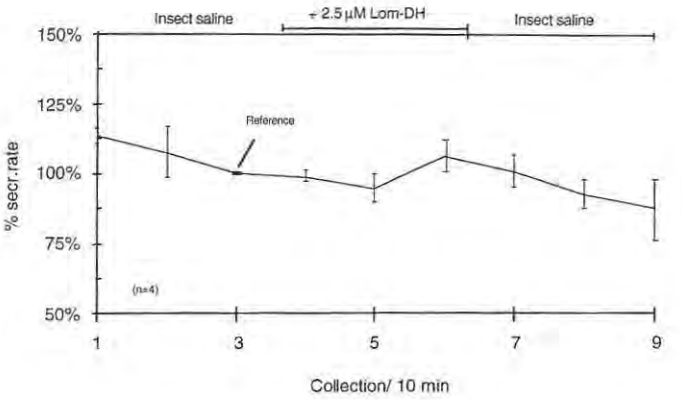

Figure 5.4. Effect of *Locusta migratoria* diuretic hormone (Lom-DH) on the fluid secretion rate of single isolated Malpighian tubules of the forest ant *Formica polyctena*. The presence of the hormone in the bath, is indicated by the bars. Mean values ± S.E.M. (n=number of tubules tested).

Cyclic AMP generating activity of the chromatographic fractions

To find out whether the chromatographic fractions expected to contain a CRF-like factor were able to stimulate cAMP production, we used a high sensitivity ^{125}I-cAMP competitive binding assay. As for the fluid secretion assay, the lyophilised chromatographic fractions were redissolved in insect saline 20 min prior to being tested in the cAMP assay. Fractions were tested at a final concentration of 20 ant-eq/µl in insect saline containing 10^{-4}M IBMX, a phosphodiesterase inhibitor.

The average protein content for Malpighian tubules from 3 ants was 9.80 µg ± 0.96 (n=20), and the cAMP level in control tubules incubated in 10^{-4}M IBMX was 45.17 ± 6.26 fmol/3 ant-eq (n=10) or, expressed per µg protein, 4.61 ± 1.09 fmol/µg protein. For the methanol extract (Fig. 5.3B) only one fraction had a clear effect on intracellular levels of cAMP. This fraction eluted between 40 and 42 min, and increased the cAMP concentration to 253 ± 9 % (n=2) compared with the 10^{-4} M IBMX controls. Some cAMP

generating activity was also found in two other fractions, one eluting between 38 and 40 min (172 ± 1%; n = 2) and the other eluting between 46 and 48 min (169 ± 6 %; n=2).

More pronounced effects on cAMP production were seen with the TFA extract (Fig. 5.3C) when tested at the same concentration (20 ant-eq/µl). The fraction eluting between 36 and 38 min increased intracellular levels of cAMP to 305 ± 1 % (n=2) compared with the IBMX controls. Some activity was also present in the neighbouring fraction eluting between 38 and 40 min, which stimulated cAMP production to 148 ± 14 % (n=2). None of the other fractions had any effect on cAMP production compared with the 10^{-4}M IBMX controls. Thus in both the fluid secretion assay and the cAMP assay the presence of activity shifted to less hydrophobic fractions for the TFA extract compared to the methanolic extract.

Presence of immunoreactive material in the head / thorax extract

An ELISA was used to demonstrate the presence of immunoreactive material in the 40% CH_3CN fraction from the 15% TFA head / thorax extract. Polyclonal antisera to Lom-DH and MAS-DH responded to a dilution series of the prepurified fraction. The overall response obtained with the polyclonal antiserum raised against Lom-DH was smaller than that obtained with the anti-Mas-DH antiserum (Fig. 5.5A), but when expressed as a percentage of the response to 1 pmol Mas-DH, used as an internal control, both antisera recognised material in the head/thorax extract to the same extent (Fig. 5.5B). There is a clear dilution pattern in the ELISA, indicating that we are dealing with a specific response of antibodies towards CRF-like material in this extract.

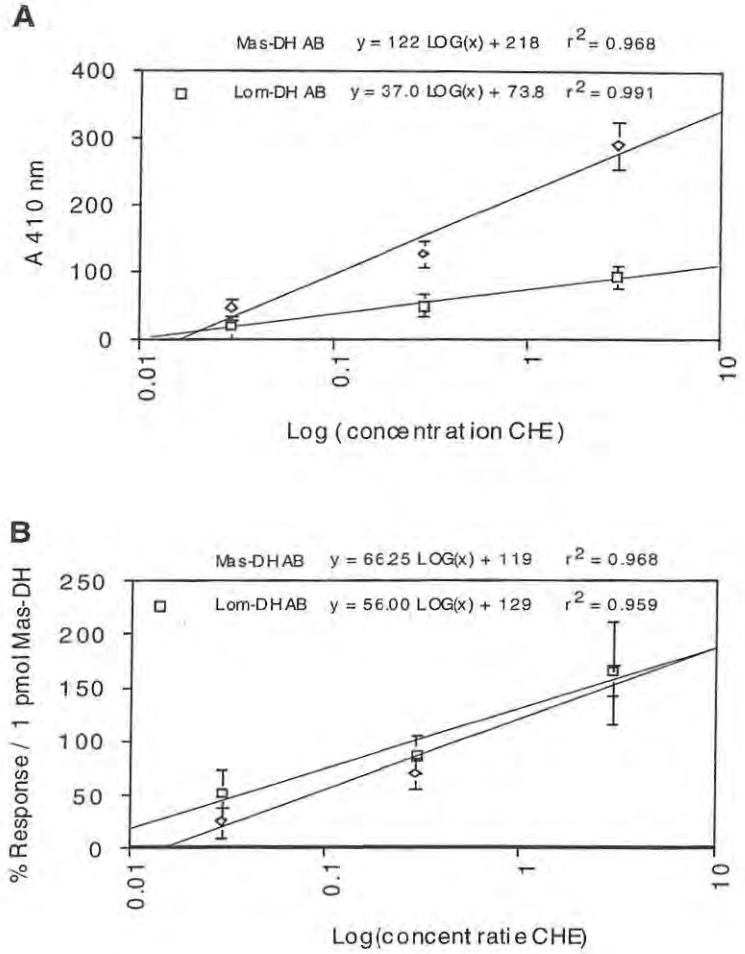

Figure 5.5. A. Immunological response of Lom-DH polyclonal antiserum (Lom-DH AB) and of Mas-DH polyclonal antiserum (Mas-DH AB) to a dilution series of the 40% CH_3CN fraction of a 15% TFA head / thorax extract (CHE). Results are expressed as the absorbance at 410 nm, mean values ± S.D., n = 2 .

B. Same results as in A but normalized to the immunological response of the polyclonal antisera to 1 pmol of synthetic *Manduca sexta* diuretic hormone (Mas-DH). (mean values ± S.D., n = 2)

DISCUSSION

CRF-like material is present in both TFA and acidic methanol head / thorax extracts of the ant

We compared acidic methanol with 15% TFA as solvents for the extraction of a CRF-related diuretic peptide from ant heads / thoraces. Acidic methanol has previously been employed in the extraction of these peptides from a number of insects (Blackburn et al., 1991),(Kay et al., 1992), (Kay et al., 1991), whereas De Decker *et al.* (1993) (De Decker et al., 1993) had shown diuretic activity to be present in a TFA extract of ant heads/thoraces. Both extracts were shown to significantly increase fluid secretion by isolated Malpighian tubules and could therefore be used as starting material for the isolation of an ant CRF-related peptide. Following the initial extraction in acidic methanol or TFA, the material was prepurified using custom-made C_4 cartridges eluted with increasing concentrations of CH_3CN. CRF-related peptides generally elute in the 40% fraction (Table 1), and this fraction had diuretic activity, although the response obtained with the methanolic extract was quite variable. When the two extracts were run on an analytical C_8-column using the same number of ant-eq, they clearly differed from one another in the amount of material present. This difference in yield must be due to the extraction procedure used. The acidic methanol procedure was more elaborate to perform and this solvent will extract membrane-associated lipids in addition to water-soluble material (Tamura et al., 1988). For example, methanol has been used for the extraction of methoxy fatty acid methyl esters from the pheromone gland of *Spodoptera littoralis* (Navarro et al., 1997) and 15% methanol was shown to be the best extraction solvent for the retrieval of prostaglandins and leukotrienes as determined by HPLC (Powell, 1987). It is therefore likely that the acidic methanol extract will contain membrane lipids. The effect of this was that during the first lyophilisation step, done to remove the methanol from the crude extract, the frozen solution changed into a viscous gel, suggesting that a substantial amount of lipid is present. This would probably hinder peptide transfer to the water phase, and could explain the low yield from this extract. This change in constitution was not observed during the lyophilisation step with the 15% TFA extract. With this acidic aqueous extraction solvent, the larger water soluble proteins are precipitated, while smaller proteins and water soluble peptides less sensitive to pH denaturation are recovered.

CRF-like peptides are present in the head / thorax extracts and co-elute with Lom-DH

In order to identify where a CRF-like peptide would elute on an analytical C_8-column, Lom-DH was used as a reference peptide. The use of Lom-DH as a reference to assist in the isolation of an ant CRF-related peptide is justified for the following reasons. Firstly, the 40% CH_3CN prepurified fraction of a 10% TFA head / thorax extract from the ant stimulated both fluid secretion and cAMP production by *Locusta* Malpighian tubules, which suggested that a peptide similar to Lom-DH was present in the ant (Laenen et al., 1998). Secondly, in this study, we have shown that material immunoreactive to polyclonal antisera raised against Lom-DH and Mas-DH is present in the head/thorax extract. Thirdly, the elution of Lom-DH in 32% CH_3CN was typical of CRF-related diuretic peptides (Table 1). This enabled us to restrict the number of fractions that needed to be assayed for biological activity using ant Malpighian tubules. Fractions with retention times of between 36 and 46 min, similar to that of Lom-DH (40-41 min) were tested for their diuretic and cAMP generating activity, two characteristic features of all insect CRF-related peptides (Coast et al., 1994). Fractions eluting between 38 and 42 min from the TFA extract significantly stimulated primary urine production, whereas in the methanol extract only the 40-42 min fraction was active. In the cAMP bioassay, fractions from the methanol extract eluting between 38 and 42 min, were the most potent, which is similar to the elution time of Lom-DH. However, for the TFA extract, the peak activity in the cAMP assay shifted to a slightly lower retention time (36-38 min). The reason for this is unclear, but the difference (<2 min) represents less than a 0.8% difference in the concentration of CH_3CN.

In view of the similarity in the retention times of CRF-like material in the ant head/thorax extract and Lom-DH and the fact that the ant extract stimulated locust tubules, it was surprising that Lom-DH had no effect on ant tubules. Mas-DH, on the other hand, almost tripled the rate of fluid secretion by ant Malpighian tubules albeit at a very high (10 µM) concentration (De Decker, 1993). Such differences in cross-reactivity most likely reflect differences in the receptors on locust and ant tubules and have been noted in other cross-species assays of insect CRF-related peptides. For example, Mas-DH had no effect on *Locusta* tubules, whereas Lom-DH was a potent stimulant of cAMP production by *Manduca* tubules. Similarly, *Tenebrio*-DH was active only at very high concentrations on *Manduca* tubules, whereas in the reverse assay Mas-DH was a potent stimulant of *Tenebrio* tubules.

In conclusion, a previous study had shown that a factor(s) with diuretic activity present in an ant head / thorax extract is destroyed by aminopeptidase M and is therefore likely to be peptidergic (De Decker et al., 1993). After fractionation on a semi-preparative BioRad Hi-pore RP 318 column, one peak containing a factor(s) with significant diuretic activity was found, using a cross-reactivity assay on *Acheta* Malpighian tubules (De Decker et al., 1993). This material eluted from the column at 29 - 34 % CH_3CN which was comparable to the values reported for CRF-related diuretic peptides in other insects (Table 1). Here we demonstrate that head / thorax extracts contain a CRF-related peptide that is able to stimulate fluid secretion and cAMP production by ant tubules. When fractionated using an analytical C_8-column, this material has a similar retention time to Lom-DH, and it is recognised by antisera raised against CRF-related peptides from *Locusta* and *Manduca*. This provides the first evidence for the presence of a CRF-related peptide in a hymenopteran insect. It suggests that the purification of *F. polyctena* diuretic hormone could best be achieved using a 15% TFA extract. The chromatographic fractions could be monitored for biological activity using a cAMP assay and/or for immunoreactivity using an ELISA and an antiserum against Mas-DH.

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CHAPTER 6

A cAMP generating peptide from the forest ant, *Formica polyctena*, was isolated and identified using cross-reactivity with the migratory locust, *Locusta migratoria*.

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Running head : A cAMP generating peptide : purification and cross-reactivity

ABSTRACT

After prepurification of a 10% trifluoroacetic acid (TFA) head extract of *F. polyctena* over Vydac 214TP C4 custom made columns, cAMP generating material was shown to be present in the 40% acetonitrile (CH_3CN) fraction. The fraction tripled the cAMP production and doubled the fluid secretion rate in single isolated Malpighian tubules of the ant.

The cAMP generating peptide was purified from the 40% CH_3CN fraction by means of a four step reversed-phase chromatography protocol. The three bioassays, used to screen the chromatographic fractions, were: the cAMP protein binding assay performed on Malpighian tubules of *Locusta migratoria*, fluid secretion experiments on tubules of *F. polyctena* and on *L. migratoria* and finally the tubule writhing assay tested on tubules of *L. migratoria*. Only fractions responding positively to all 3 tests were further purified. The tubule writhing assay was fast and easy to perform. It was used as a first indication for the presence of physiologically active material.

This screening procedure finally lead to the purification of a peptide with a molecular mass of 2,966 daltons. The full sequence of the peptide could be obtained in one single run and was : GLDLG LSRGF SGSQA AKHLM GLAAA NYAGM. A computer search of sequence data banks did not reveal any significant similarity of this peptide with other known insect diuretic peptides. This 30mer peptide, called Fop-cGP, did not belong to the corticotropin releasing factor family of insect diuretic peptides as might have been inferred from its cAMP generating and secretion stimulating effect. It had a myotropic effect on the muscle fibres of the Malpighian tubule of *L. migratoria* and tripled both the cAMP-production and the fluid secretion rate. On Malpighian tubules of *F. polyctena* only its effect on the fluid secretion rate has been tested. Fop-cGP had only a small diuretic effect on the primary urine production, which may indicate that Fop-cGP is not the prime stimulator of fluid secretion in worker ants. So the actual physiological role in the ant still needs to be investigated.

Keywords :
Malpighian tubules, primary urine production, tubule writhing assay, neuropeptide

INTRODUCTION :

In all insects studied so far the primary urine production seems to be regulated by means of diuretic neuropeptides (Gäde et al., 1997),(Panabecker et al., 1993),(Phillips, 1982),(Spring, 1990). Two peptide families were recognised which seem to work synergistically in regulating the fluid secretion rate in insect Malpighian tubules (Spring and Clark, 1990),(Coast, 1996),(Maddrell et al., 1993). The first family is the family of myokinins named after their myotropic effect on the hindgut of the cockroach *Leucophea madeira* (Schoofs et al., 1993). They are small peptides (6 - 13 amino acids) with a highly conserved C-terminal core sequence. The second family of insect diuretic peptides showed some homology with the human corticotropin releasing factor (CRF) / sauvagine / urotensin-I family of vertebrate peptides and was consequently named the CRF-related family of diuretic peptides (Coast et al., 1994). They are much larger (30 - 46 amino acids) and no clear core sequence could be recognized. Structure-activity studies showed that the C-terminus might be important for receptor binding and that the opposite end (NH_2-terminus) is involved in signal transduction (Nachman et al., 1995),(Reagan, 1995). Both families use a distinct signal transduction mechanism. The myokinins act through an increase in intracellular calcium and were shown to reduce the intercellular resistance by increasing the Cl^- conductance (Montuenga et al., 1996). The CRF-related peptides bind to G-protein coupled receptors and activate adenylate cyclase which results in an increase in the intracellular cAMP concentration (Reagan, 1994),(Reagan et al., 1993).

In the ant, up to now, two myotropic peptides have been characterized (De Decker, 1993). During the purification of these peptides, chromatographic fractions with both diuretic and myotropic activity, were shown to be present (De Decker, 1993), which suggest that myokinins might be present in head extracts of the ant.

Furthermore diuretic factors are present in head extracts of the ant (De Decker et al., 1994) and a diuretic factor, named *F. polyctena* diuretic peptide (Fop-DP), was partially purified from the 20% CH_3CN fraction of a 10% TFA head / thorax extract (Laenen et al., 1998b). It stimulated the primary urine production and depolarised the apical membrane potential of the ant Malpighian tubules. Fop-DP had a molecular mass of 7,514 daltons however and may therefore not be related to the CRF-like peptide family. Also Fop-DP had no effect on the cAMP production and probably acts via a calcium dependent mechanism (Laenen et al., 1998b).

In earlier experiments head extracts of *F. polyctena* were shown to stimulate the cAMP production and the primary urine production in isolated Malpighian tubules of the ant

(Laenen et al., 1998a). Possibly, another factor, different from Fop-DP was responsible for these effects. Immunological methods indicated that CRF-like material was present in these extracts. In addition, diuretic and cAMP stimulating material from these extracts elutes close to Lom-DH when fractionated on an analytical C8 column. These results suggested the presence of CRF-related material in ant head extracts. In the present study a 10% TFA extract was made of a large number of ants (300,000) and a further attempt was made to isolate this CRF-related material.

MATERIALS AND METHODS

Insects

The ants used in the experiments were collected from their natural nest in the forest of Slederlo (Genk, Belgium). They were reared at 25°C and 65% relative humidity under a 16h:8h light:dark cycle, and fed on a diet of sugar and water. Adult *L. migratoria* were taken from the existing colony at Birkbeck College.

Preparation of a 10% TFA head/thorax extract

A 10% TFA head/thorax extract was prepared from 300,000 ants as described previously (De Decker *et al.* 1994). The crude extract was fractionated on activated custom-made 4cc Vydac 214TP C4 syringe cartridges (Varian). Batches of 2,500 ant-equivalents (ant-eq) were loaded per cartridge. Each cartridge was stepwise eluted with 90 ml aliquots of increasing acetonitrile (CH_3CN) concentration (20, 40, 65 and 80%). The separate CH_3CN fractions were lyophilised separately in a Heto-Vac system and stored in the freezer at -70 °C until further use.

Bioassays

Screening of the fractions obtained in the different purification steps was performed using the following bioassays. Essential in the present study was the assessment of the stimulation of cAMP by isolated Malpighian tubules of the ant. The tubules of three animals (i.e. ca 45 tubules in all) were pooled and preincubated for 30 minutes at room temperature in 35 µl of insect saline (composition mM : 27.0 KCl, 8.04 K_3citrate, 1.97 $CaCl_2$, 13.0 $MgCl_2$, 16.8 Na_2fumarate, 14.4 Na_2succinate, 2.80 L-alanine, 10.5 trehalose, 11.7 maltose, 12.1 Hepes and 138.8 glucose. The saline had an osmolality of 350 mosm/kg and was adjusted to a pH of 7.2 by means of NaOH), it

contained 100 µM isobutylmethylxanthine (IBMX), a phosphodiesterase inhibitor. After preincubation, 15 µl of a test solution was added. This test solution consisted of 15 ant-eq of the 40% acetonitrile fraction redissolved in 50 µl of *Formica* saline containing 100 µM IBMX. After a second incubation of 30 min at room temperature the reaction was stopped by adding 900 µl of ice-cold methanol and an incubation on ice for 15 minutes. Formed cAMP was liberated from the cells by sonication (3 times 30 seconds) in a sonication bath. The reaction vials were centrifuged for 15 minutes in a eppendorf centrifuge and the supernatant containing the cAMP was transferred to a new eppendorf tube and dried. The cAMP content of the lyophilised material was determined by means of a ^{125}I cAMP scintillation proximity assay system, according to the instructions supplied by the manufacturer (Amersham International, RPA538). The protein content of the pellets was determined by means of a BCA-protein determination kit (Pierce), using bovine serum albumin (BSA) as a standard.

The difficulty of working routinely with *Formica* Malpighian tubules (in the fluid secretion assay only one tubule can be tested at a time) lead us to investigate the use of tubules from other insects in the screening of chromatographic fractions. The effect of the 40% CH_3CN fraction of a head/thorax extracts of *Formica* on the cAMP production by Malpighian tubules of some species (table 1), was investigated by means of a previously described, competitive binding assay (Wheeler and Coast, 1990). The response was most marked in the *L. migratoria* tubules and this species was consequently used to screen for biological activity in the chromatographic fractions.

An additional benefit of this clear cross-reactivity with *Locusta* tubules was the possibility to use the tubule writhing assay . It has been observed that the CRF-related peptides increase the frequency of spontaneous contractions of the spiral muscle layer around the Malpighian tubules of *Acheta domesticus, Periplaneta americana, L. maderae* and *L. migratoria* (Coast, 1998). As a consequence the tubule writhing assay could be used to screen for CRF-related peptides in the chromatographic fractions obtained for the ant head / thorax extract. In this assay a locust tubule is fixed by two holding pipettes in an incubation chamber continuously perfused with *Locusta* saline (composition mmol/l : 5.0 K_2SO_4, 10.0 $MgSO_4$, 4.0 $NaHCO_3$, 5.0 $CaCl_2$, 100.0 NaCl, 14.4 Na_2succinate, 2.9 L-alanine, 13.1 proline, 6.5 serine, 1.0 tyrosine, 1.8 valine, 1.4 histidine, 1.4 lysine, 11.4 glycine, 5.0 glutamine, 1.3 asparagine, 1.0 arginine, 25.0 hepes and 10.0 glucose, the pH was adjusted to 7.2 by means of NaOH). One of the holding pipettes is made from flexible tubing and holds a small flag. A binocular microscope (Willovert) connected to a photomultiplier tube (pmt) is focussed on this flag as shown in figure 6.1A. The changes in light intensity, due to the tubule contractions, are detected by the pmt and translated into an electrical signal recorded on a strip chart recorder. The high sensitivity of this assay allowed to screen the HPLC-fractions directly without pretreatment, which facilitated the search for biological activity during the purification process. Two µl aliquots of the chromatographic fractions were diluted in 1 ml of *Locusta* saline and pumped directly into the incubation chamber.

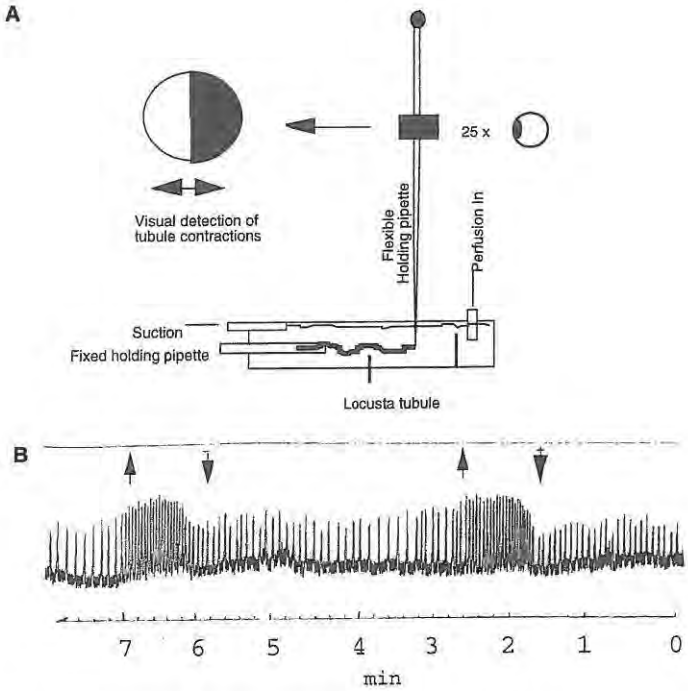

Figure 6.1 A. Experimental set-up for the *Locusta* tubule writhing assay
B. Representative trace for the myotropic response of the *Locusta* tubules to 0.001 ant-eq/µl of the 40% CH_3CN fraction of a 10% TFA head / thorax extract of the ant.
The timescale reads from right to left. Adding (↓) and wash-out (↑) of the test substance is indicated by the arrows. Effect is shown twice.

Chromatographic fractions which promoted both tubule writhing and cAMP production in single isolated *Locusta* tubules were tested in the fluid secretion assay. Fluid secretion assays were performed on both *Locusta* tubules and *Formica* tubules, as described in (Coast, 1998) and (De Decker et al., 1994) respectively. If all three conditions were fulfilled, promoting tubule writhing, increase in cAMP production and increase in fluid secretion rate, the fraction was used for the next purification step.

Purification

High-performance liquid chromatography (HPLC) was performed on a Waters equipment consisting of two 510 HPLC-pumps, a 486 tunable U.V./Visible absorbance detector set at 215 nm and the millennium 2.15 chromatography manager. Four chromatographic steps were needed to obtain a purified, biologically active peptide. In a first step approximately 50,000 antequivalents (ant-eq) of the lyophilised material of the 40% CH_3CN fraction were redissolved in 50 ml of 5% CH_3CN, 0.1% TFA and loaded on a semi-preparative Vydac C8 column (10µm, 300Å, 250x10 mm) in successive injections of 5ml. Chromatographic fractions were obtained using a linear gradient of 0 - 60% CH_3CN in constant 0.1% aqueous TFA over 60 min. A flow rate of 2 ml/min was employed and fractions were collected at 2 min intervals. In total 6 runs were needed to process the total crude extract.

Secondly, bioactive fractions were reduced in volume to approximately 1ml, diluted with 4 ml of 0.1% TFA, and applied to an analytical C8 column (Aquapore RP-300, 7 µm, 300Å, 240x4.6 mm). A linear gradient of 20 - 60% CH_3CN was applied over 50 min in constant 0.1% aqueous TFA, flow rate was 1 ml/min. Peak fractions were collected manually.

Thirdly, fractions with biological activity were treated as described above and subsequently bound to a Vydac Diphenyl column (5µm, 300Å, 250x4.6 mm). The material was eluted from the column using the same solvents and conditions as for the previous column. Peak fractions were collected manually.

Finally the active peak fraction was rechromatographed on the analytical C8 column using the same conditions as before and yielded a single U.V.-absorbing peak.

Mass spectrometry and amino terminal sequencing

The mass of the purified peptide was determined on a TRIO-2 quadropole spectrometer (VG Masslab Ltd.) fitted with an electrospray source (VG Biotech Ltd.). Electrospray mass-spectrometry was carried out according to the protocol recommended by the manufacturer. Amino acid sequence information was obtained by means of Edman degradation on an Applied Biosystems 477A sequencer employing rapid cycles.

Statistics

Results are given as mean values ± standard error (S.E.M.) with the number of tubules in parentheses. Statistical significance was calculated using the paired Student's *t*-test, unless indicated otherwise. A difference was considered significant if $p \leq 0.05$.

RESULTS

Biological activity of the 40% CH_3CN fraction

The 40% acetonitrile fraction was tested on the ant tubules both in the fluid secretion assay (Fig. 6.2) and in the I^{125}-cAMP competition assay of Amersham. The diuretic effect on the fluid secretion rate, confirmed the observations done for the 40% CH_3CN fraction of a 10% TFA head/thorax extract (Laenen, Coast, 1998) (see also Chapter 3 p.70). At a test concentration of 0.1 ant-eq/µl, the 40% CH_3CN fraction doubled the fluid secretion rate (Fig. 6.2). The same test concentration was used in the I^{125}-cAMP assay and resulted in a threefold increase of the intracellular cAMP production to 300% ± 23 (n=6, $p \leq 0.05$) compared to the control in the presence of alone 10^{-4}M IBMX.

Table 1 : *cAMP production in Malpighian tubules of different insect species, in response to the 40 % CH_3CN fraction of a 10 % TFA head / thorax extract of the forest ant.*

Species	control 0.6% CH_3CN	40% CH_3CN fraction 0.3 ant-eq/µl	forskolin 5.10^{-4} M
Locusta migratoria	1.85 ± 0.87	20.91 * ± 5.47	35.96 * ± 12.14
Acheta domesticus	11.80 ± 8.80	14.56 ± 10.11	27.37 * ± 6.11
Leucophea maderae	0.45 ± 0.04	4.92 * ± 1.81	24.28 * ± 3.48
Manduca sexta	0.74 ± 0.24	0.45 ± 0.15	1.79 ± 0.57

Values represent pmol cAMP ± S.E.M. (number of assays = 3). * Significantly different from control ($p \leq 0.05$, unpaired Student's *t*-test).

Furthermore this fraction was used at a test concentration of 3 ant-eq/µl in the competitive binding assay to assess the effect on the intracellular cAMP production in Malpighian tubules of other insect species (Table 1). Only Locust tubules responded to the ant's extract in more or less the same way as they responded to 5.10^{-4}M forskolin, an adenylate cyclase activator. This clear cross-reactivity of the ant extract with locust tubules was confirmed in the tubule writhing assay. A test concentration of 0.001 ant-eq/µl was sufficient to see a clear stimulating effect (Fig. 6.1B).

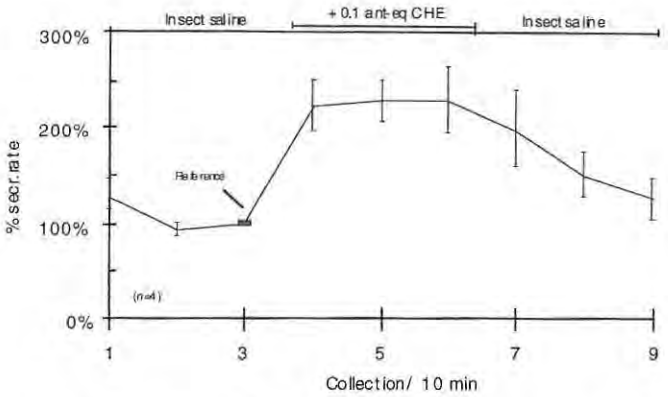

Figure 6.2 Effect of the 40% CH_3CN fraction, tested at a concentration of 0.1 ant-eq/µl, on the fluid secretion rate in single isolated Malpighian tubules of the forest ant *Formica polyctena*. The secretion rates are expressed as a % of secretion rate at the third control period. The presence of the 40% CH_3CN fraction in the bath is indicated by the bar. Mean values ± S.E.M., (n=number of tubules tested).

Purification of Fop-cGP

From the fractions collected every 2 min from the semi-preparative Vydac C8 column (Fig. 6.3A) one active region around fraction 40 could be detected in the cAMP competitive-binding assay (Table. 2). This activity was confirmed in the fluid secretion assay for both insect species. For the locust tubules the diuretic activity was confined to fraction 40, whereas for the ant tubules diuretic activity was found in fraction 39 as well (Table 2). The most specific response was obtained in the tubule writhing assay. All the chromatographic fractions were tested but only one, fraction 40, was active in the *Locusta* tubule. Tested at a concentration of 0.025 ant-eq/µl it had a clear myotropic effect as can be seen in a representative trace (Fig. 6.3B).

Table 2 : *Screening results for the chromatographic purification of Fop-cGP*

	Species	*Locusta migratoria*				*Formica polyctena*			
HPLC - column	Assay	Fraction N°				Fraction N°			
Vydac C8		38	39	40*	41	38	39	40*	41
	cAMP production	3.18	5.35	12.45	5.44	---	---	---	---
	Fluid secretion	101 ± 18	105 ± 14	154 ± 14†	128 ± 15	93 ± 14	143 ± 19†	144 ± 3†	124 ± 11
Aquapore C8		14	15	16*	17*	14	15	16*	17*
	cAMP production	2.20	3.71	15.06	6.08	---	---	---	---
	Fluid secretion	---	---	---	---	---	---	133 ± 9†	---
Vydac diphenyl		23	24*	25*	26	23	24*	25*	26
	cAMP production	1.17	3.29	12.60	0.66	---	---	---	---
	Fluid secretion	65 ± 11	113 ± 15	149 ± 24†	84 ± 14	---	---	129 ± 1†	---
Aquapore C8		1	2*	3	4	1	2*	3	4
	cAMP production	1.52	6.76	2.47	1.16	---	---	---	---
	Fluid secretion	75 ± 10	329 ± 24†	126 ± 11	101 ± 11	---	130 ± 2†	---	---

* fractions with myotropic activity in the locust tubule writhing assay. --- : No Data Available
cAMP production values represent average amount of pmol cAMP determined for two samples.
Fluid secretion of Malpighian tubules represent fluid secretion rates, expressed as a % of the internal control.
† Significantly different from the internal control (paired Student's *t*-test, $p \leq 0.05$).
Mean values ± S.E.M. , number of tubules tested for the locust = 6, for the ant = 2.

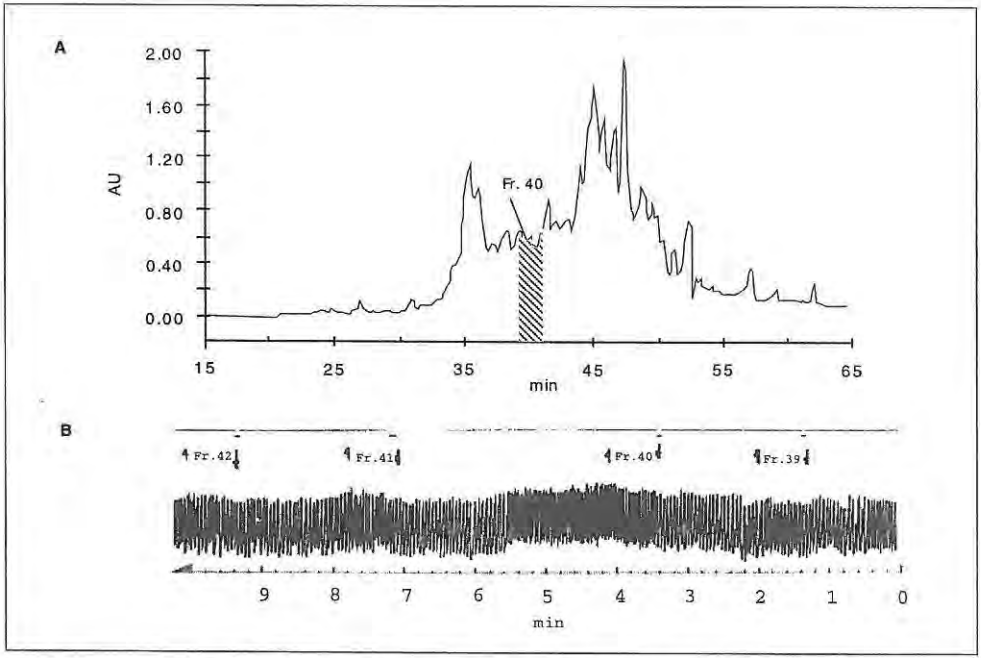

Fig. 6.3 A. U.V. absorbance profile of 50,000 ant-eq of the 40%SepPak fraction separated on a Vydac C8 column (10µm, 300Å, 10x250 mm). Conditions are described in Materials and Methods. The marked area contains the fraction with myotropic activity on single isolated *Locusta* tubules.

B. Representative trace for the myotropic response of the *Locusta* tubules to 0.025 ant-eq/µl of the chromatographic fractions from the Vydac C8 column. Adding (↓) and wash-out (↑) of the test substances is indicated by the arrows.

Fraction 40 and the neighbouring fractions of the preparative runs were pooled, reduced in volume and applied to an analytical Aquapore C8 column. In total three runs were needed to process the material. In the tubule writhing assay the response was spreading from the shoulder fraction on through the major peak in the chromatogram (Fig. 6.4).

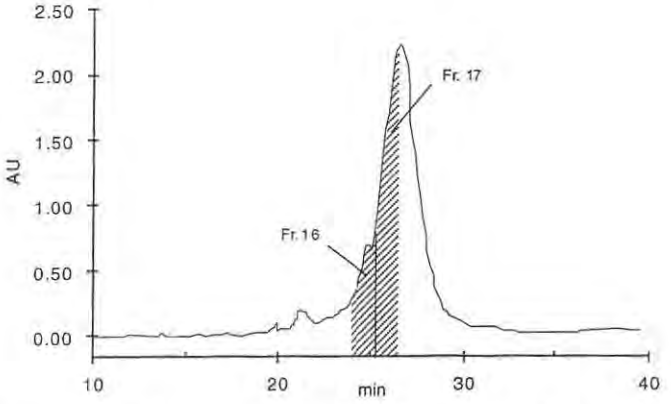

Figure 6.4 U.V. absorbance profile of 100,000 ant-eq of fraction 40 separated on an Aquapore C8 column (7µm, 300Å, 240x4.6 mm). Conditions are described in Materials and Methods. The marked areas are fractions with myotropic activity on single isolated *Locusta* tubules

In the cAMP assay the activity was mainly constricted to the shoulder fraction (fraction 16) (Table 2). The diuretic activity of fraction 16 was confirmed in fluid secretion assays performed only on single isolated Malpighian tubules of the ant. Tested at a concentration of 0.6 ant-eq/µl the shoulder fraction stimulated the fluid secetion rate (Table 2).

Since this HPLC-step did not result in a highly resolved chromatogram, the column chemistry was changed for the next chromatographic fractionation. Changing from an alifatic to an aromatic diphenyl charged column (Vydac diphenyl, 300Å, 250x4.6 mm), managed to spread the material over 14 fractions (Fig. 6.5).

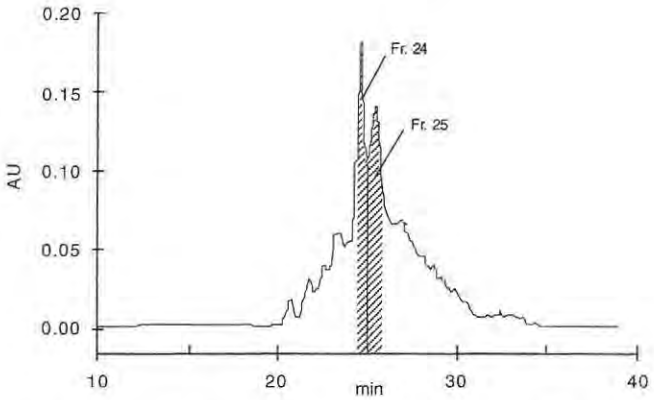

Figure 6.5 U.V. absorbance profile of 300,000 ant-eq of fraction 16 separated on a Vydac Diphenyl column (7µm,300Å,4.6x250mm). Conditions are described in Materials and Methods. The marked areas are fractions with myotropic activity on single isolated *Locusta* tubules

At first sight the tubule writhing assay showed more activity in fraction 24, but after a 1 in 10 dilution the activity could only be recovered in fraction 25, a result confirmed both in the fluid secretion and the cAMP assay (Table 2).

This bioactive material, i.e. fraction 25, was rechromatographed on the analytical C8 column in order to find out whether it corresponded to the activity seen in fraction 16 from the aquapore C8 column. The same gradient conditions were used as before, only the sensitivity of the detector was changed to a full range value of 0.150 AU. Instead of one single peak, two separated peaks named fraction 2 and 3 were recovered (Fig. 6.6A). All the biological activity was confined to fraction 2.

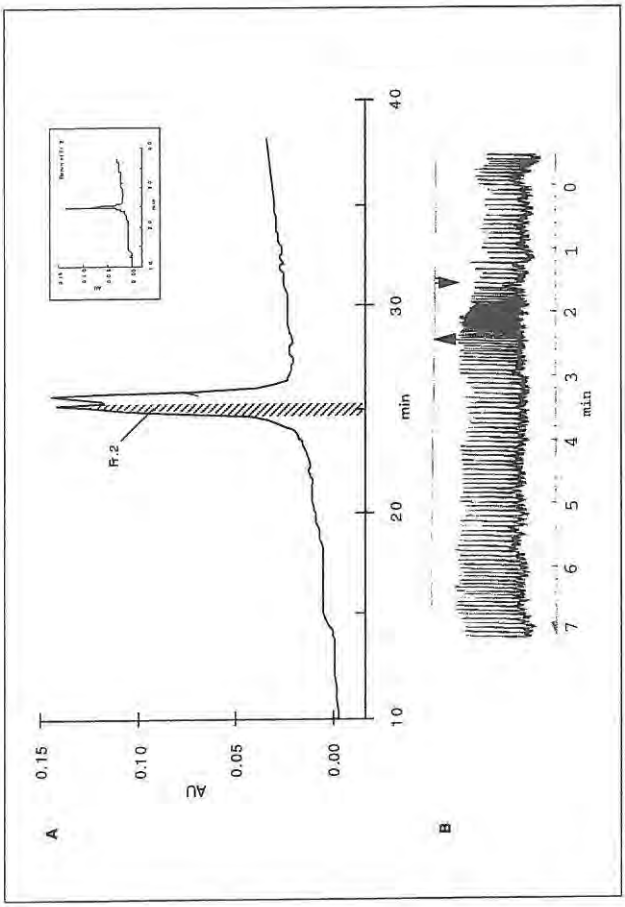

Fig. 6.6 A. U.V. absorbance profile of 300,000 ant-eq of fraction 25 separated on an Aquapore C8 column (10µm,300Å,10x250mm). Conditions are described in Materials and Methods. The marked areas are fractions with myotropic activity on single isolated *Locusta* tubules Inset: rerun of Fr. 2, same conditions as in A.

B. Representative trace for the myotropic response of the *Locusta* tubules to 0.025 ant-eq/µl of peak fraction 2 from the Aquapore C8 column. Adding (↓) and wash-out (↑) of the test substances is indicated by the arrows.

Mass and primary structure of the purified peptide

Approximately 30,000 ant-eq of fraction 2 were rechromatographed on the analytical C8 column (Inset Fig. 6.6A), lyophilised and used for mass spectrometry and aminoterminal sequencing. The native purified peptide did not need a treatment with aminopeptidase-M prior to the Edman degradation. This indicated that the amino terminus was not blocked with pyroglutamate (Pan et al., 1984). The sequence of the first 29 amino acids could be obtained in one single run. To determine the mass of the peptide, a sample was subjected to electrospray mass-spectrometry. This yielded a mass of 2966 Da. which was 131.25 Da

havier than the mass calculated for the first 29 amino acids. Respecting the loss of one water molecule for binding to the C-terminal amino acid, an expected mass for this amino acid can be calculated to be 149.25 Da. This is the mass of methionine and as such the full sequence of Fop-cGP reads as follows: GLDLG LSRGF SGSQA AKHLM GLAAA NYAGM.

Biological activity of the purified peptide

The purified peptide had the following effects on *L. migratoria* Malpighian tubules. Tested at a concentration of 0.5 ant-eq/µl it tripled both the fluid secretion rate and the intracellular cAMP concentration (Table 2). Most potent was its myotropic effect, a test concentration of only 0.025 ant-eq/µl was sufficient to evoke a stimulatory effect on the spontaneous contractions of the locust Malpighian tubules in the tubule writhing assay (Fig. 6.6B). For the *F. polyctena* Malpighian tubules only the effect on the primary urine production was measured. Tested at a concentration of 1.0 ant-eq/µl the petide stimulated the fluid secretion rate to 130% ± 2 (n=2, p≤0.01).

DISCUSSION

Biological effect of the crude extract

The 40% CH_3CN fraction of a 10% TFA head/thorax extract had a stimulatory effect, both on the primary urine production and on the cAMP production of the ant Malpighian tubules. This activity, diuresis mediated through cAMP as second messenger, is typical for the CRF-related peptides (Coast et al., 1994),(Gäde et al., 1997) and suggested the presence of one of these factors in the extract. In a previous study this extract was shown to contain immunologically reactive material, using polyclonal antisera directed against Lom-DH or *Manduca sexta* diuretic hormone (Chapter 5). It is generally accepted that CRF-related peptides are major diuretic factors in insects. In the present study an attempt was made to characterize such a factor in a species of the order of the Hymenoptera for the first time.

Bioassays used for screening of the chromatographic fractions

Only a small number of ants, approximately 100, were available in the laboratory at Birkbeck College. For a standard cAMP assay on the ant tubules, 50 to 60 ants were needed. To screen for biological activity in the chromatographic fractions it was consequently necessary to use cross-reactivity with insects available in London.

Cross-reactivity has been used previously with success for the purification of diuretic peptides from *P. americana, Musca domesticus, Stomoxys calcitrans and M. sexta* (Clottens et al., 1994), (Kataoka et al., 1989),(Kay et al., 1992). The choice of the bioassays was based on the known physiological effects of the CRF-related peptides known to exist in other insects. CRF-like peptides are known to increase fluid secretion in Malpighian tubules via cAMP (Audsley et al., 1995),(Coast et al., 1994). Moreover it was observed that the CRF-related peptides increase the frequency of the spontaneous contractions of the Malpighian tubules in *Acheta domesticus, P. americana, L. maderae* and *L. migratoria* (Coast, 1998). This myotropic effect on its own, is not the cause of the observed diuretic effect. The stimulation of the primary urine production was still present in high potassium saline in which the spontaneous contractions stop (Coast, 1998). In addition, some myotropic peptides such as the locustatachykinins have a very potent myotropic effect, but they have no effect on the observed fluid secretion rates (Schoofs et al., 1993). Consequently the locust tubule writhing assay can be used as an assay to detect biological active material, but was insufficient on its own to screen the chromatographic fractions for the presence of diuretic factors. Supplementary information was obtained from effects on fluid secretion of the Malpighian tubules and from the cAMP competitive binding assay (Wheeler and Coast, 1990).

Purification and characteristics of Fop-cGP

The combination of the three assays lead to a rapid and highly specific screening of the HPLC fractions. A better resolution in chromatography was obtained on changing from an alifatic to an aromatic stationary phase. This change in column chemistry caused the active fraction of the previous column to spread over a large number of U.V.-absorbing fractions, but confined the biological activity to only one single fraction. Further purification of this active fraction finally resulted in a single, pure U.V.-absorbing peak.

Chemical characterization of the purified peptide was achieved on approximately 150 pmol of purified peptide (estimate of amount was based on the initial sequencing yields for the aminoterminal sequencing). Knowing that this material was purified from 30,000 ants, and assuming that no material was lost during the purification protocol, this result suggested that 1 ant contains about 5 fmol of the diuretic peptide. As a consequence the observed concentrations at which the purified peptide was active, range from 10^{-9}M - 10^{-7}M. The values reported for the CRF-related peptides range from 10^{-11}M - 10^{-9}M (Coast et al., 1994),(Coast, 1995),(Holman et al., 1990) which is two orders of magnitude lower. But the purification of the peptide was based on cross-reactivity with locusts and

Audsley *et al.* (1995) demonstrated that the Malpighian tubules of *L. migratoria* needed higher concentrations of CRF-related peptides from other insects. It is therefore to be expected that the concentration range active in the ant will be comparable to the values reported for the CRF-related peptides.

Based on the activity of the peptide, diuresis mediated through cAMP as a second messenger, it was expected to be the CRF-related peptide from the forest ant *F. polyctena.* But the primary structure, obtained through Edmann degradation, was completely different. The peptide was named *Formica polyctena* cAMP Generating Peptide (Fop-cGP). A computer search of sequence data banks did not reveal any significant similarity of Fop-cGP with other known diuretic peptides, only an irrelevant similarity with the brkA protein of *Bordetella pertussis* was obtained. BrkA is a large (1010 amino acids) transmembrane protein, involved in the resistance of *B. pertussis* to antimicrobial peptides (Fernandez and Weiss, 1996). So far Fop-cGP is the second peptide purified from insects, with an activity on insect Malpighian tubules resembling that of the CRF-related peptides, but with no structural relation to the latter (Spittaels et al., 1996).

Speculation on a possible dual control mechanism of primary urine formation in the ant Malpighian tubule and suggestions for future research

Dual control has been described for other insect species (Coast, 1995),(Coast, 1996). Best known is the synergism observed for the CRF-related peptides and the myokinins in locusts. At low concentrations, the peptides co-operatively stimulate fluid secretion to a value higher than the sum of the values they would have reached if they had been applied separately. Candidates for a dual control of primary urine production in the ant are the *F. polyctena* diuretic factor (Fop-DP) (Laenen et al., 1998b) and Fop-cGP. Fop-DP acts via calcium and Fop-cGP via cAMP, like the myokinins and CRF-like peptides respectively. Furthermore Fop-DP had an additive effect on top of that of cAMP (Laenen et al., 1998b) and vice versa. The diuretic effect of Fop-cGP alone in ant tubules was small, compared to the stimulation seen in locust tubules. This might indicate that Fop-cGP is not the prime stimulator of the fluid secretion in the worker ants. Either its most important physiological function lies elsewhere or a second peptide for instance Fop-DP must be present to obtain a maximal effect on the primary urine production.

Further experiments are needed to investigate whether Fop-cGP and Fop-DP do co-operate and play a primary role in the control of the ant primary urine production.

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SAMENVATTING

De Malpigische vaatjes van de bosmier, *Formica polyctena*, worden al verschillende jaren bestudeerd binnen de onderzoeksgroep fysiologie. Deze vaatjes zijn bij insecten verantwoordelijk voor de primaire urineproduktie. Deze laatste wordt gerealiseerd door een osmotische filtratie die aangedreven wordt door het actief transport van KCl van de hemolymfe naar het lumen. De onderzoeksgroep onderzocht eerst welke transportmechanismen, binnen de Malpigische vaatjes, verantwoordelijk zijn voor dit actief transport van KCl. In een volgend stadium werd onderzoek gedaan naar de regelingsmechanismen voor dit transport. Er werd daarbij meer bepaald gezocht naar endogene factoren met een rechtstreeks effect op de primaire urineproduktie door de Malpigische vaatjes. Zo konden in kopextracten van de mier zowel diuretische als antidiuretische factoren aangetoond worden. Enkele van deze factoren, met name een diuretisch peptide (Fop-DP I) en twee myotrope peptiden (Fop-TK I en Fop-TK II), werden opgezuiverd. In de huidige studie werd getracht dit beeld verder aan te vullen. Zo konden verschillende factoren, zowel diuretische (Fop-DP en Fop-cGP) als een antidiuretische (FopADF), in deze studie aangetoond en soms opgezuiverd worden in lichaamsextracten van de mier.

Voor de antidiuretische factor, die werd opgezuiverd uit achterlijven, kon uitgemaakt worden dat hij de actieve transportmechanismes in de Malpigische vaatjes van de mier stillegt (zie hoofdstuk 1).

Het signaaltransductiemechanisme, dat door FopADF gebruikt wordt, kon niet opgehelderd worden. Het kon in ieder geval uitgesloten worden dat FopADF een adenylaat cyclase nodig heeft, en dat het een invloed heeft op de intracellulaire cAMP concentratie (zie hoofdstuk 2). Een duidelijke kruisreactiviteit van FopADF in *Tenebrio molitor* werd aangetoond door Dr. S. Nicolson (zie Note na hoofdstuk 1) en de aanwezigheid van een gelijkaardige antidiuretische factor kon worden aangetoond in een extract gemaakt van achterlijven van de honingbij, *Apis mellifera* (zie hoofdstuk 4).

Voor de diuretische factoren, die enkel uit koppen opgezuiverd werden, kon aangetoond worden dat het over peptiden handelt. Van Fop-DP werd voor ongeveer 35% de aminozuursequentie bepaald en kon worden uitgemaakt dat het zijn diuretisch effect realiseert via een verhoging in intracellulair calcium (zie hoofdstuk 3). Ook voor Fop-DP

kon een verwante factor aangetoond worden in kopextracten van de honingbij (zie hoofdstuk 4).
De tweede diuretische factor, Fop-cGP, werd volledig gekarakteriseerd. Het is een peptide van 30 aminozuren, het heeft een diuretisch effect op de mier en de sprinkhaan en realiseert zijn effect door een verhoging in cAMP (alleen nagegaan bij de sprinkhaan) (zie hoofdstuk 6). De opzuivering van Fop-cGP resulteerde uit een vooronderzoek naar de aanwezigheid van CRF-verwante peptiden bij de bosmier. Deze laatsten konden met behulp van chromatografie en ELISA aangetoond worden in een koppenextract van de bosmier (zie hoofdstuk 5).
In wat volgt wordt getracht de resultaten, bekomen in elk hoofdstuk, beknopt weer te geven. Om te voorkomen dat de lezer de rode draad verliest, werd er een overzichtstabel, in de vorm van een bladwijzer, aan het werk toegevoegd. Hierin trachten we de mier te spiegelen aan andere insecten, inzake de regeling van de primaire urineproduktie.

Hoofdstuk 1
In het eerste hoofdstuk van de huidige studie wordt uiteengezet hoe een antidiuretische factor uit lichaamsextracten van de bosmier, *F. polyctena*, wordt opgezuiverd. Het is namelijk zo dat er vroeger reeds werd vastgesteld dat de secretiesnelheid in de Malpigische vaatjes van de bosmier sterk variabel kon zijn (Van Kerkhove et al., 1989). In sommige gevallen kon zelfs geen secretie bekomen worden. Dit laatste deed zich voornamelijk voor bij mieren die pas van de natuurlijke nest verzameld werden. Daags nadien echter waren de Malpigische vaatjes van hetzelfde lot mieren perfect bruikbaar in secretie-experimenten wat het vermoeden deed rijzen dat de bosmier factoren bezit met een rechtstreeks antidiuretisch effect op de primaire urineproduktie door de Malpigische vaatjes.
In een vorige studie kon dit inderdaad aangetoond worden voor kopextracten van de mier. Na een chromatografische fractionatie werden er fracties teruggevonden met een diuretische en/of antidiuretische activiteit (De Decker et al., 1994). In de huidige studie zijn we vertrokken van een hemolymfe extract. Deze keuze werd ingegeven door het feit dat de Malpigische vaatjes, het doelorgaan van de antidiuretische factoren, bij de mier in vrij contact met de hemolymfe in het achterlijf gelegen zijn (Wigglesworth, 1931). Verwacht wordt dat de hypothetische, antidiuretische factor(en) terug te vinden zal (zullen) zijn in de hemolymfe van mieren met niet secreterende vaatjes. De hemolymfe van zulke mieren werd verzameld en geëxtraheerd. Dit extract had een antidiuretisch effect als het werd uitgeprobeerd op secreterende vaatjes. Omdat de collectie van hemolymfevloeistof uit het

achterlijf tijdrovend is, werd een 15% trifluorazijnzuur (TFA) extract van hele achterlijven van 150,000 mieren gemaakt. Dit abdomenextract had ook een inhiberend effect in secretieexperimenten en werd gebruikt in een eerste poging om de factor te identificeren die verantwoordelijk was voor de antidiuretische respons. Na een 4 staps HPLC-protocol werd er inderdaad een chromatografisch zuivere factor gevonden met een duidelijk antidiuretisch effect op de secretiesnelheid van Malpigische vaatjes van de mier. Deze factor werd de *Formica polyctena* antidiuretische factor (FopADF) genoemd in overeenstemming met de nomenclatuur van Raina en Gäde (1988). De moleculaire karakterisatie leverde niet de beoogde resutaten op, wat te wijten kan zijn aan het feit dat we te doen hebben met een cyclisch peptide, zoals gesuggereerd wordt in de discussie. Naast het antidiuretisch effect in de secretie experimenten werd ook het effect op de membraanpotentialen onderzocht. Aangetoond werd dat FopADP in staat was én het basolateraal én het apicaal membraan te depolariseren, een respons die volledig omkeerbaar was bij uitwassen van de antidiuretische factor. Een vergelijkbare respons werd voordien reeds beschreven voor het gecombineerd effect van de metabole inhibitor dinitrophenol (DNP) en Ba^{2+}. DNP zal de actieve, elektrogene protonpomp stilleggen en Ba^{2+} is een selectieve blokker van het K^+ kanaal (Leyssens et al., 1993). Dit suggereert dat FopADP de actieve K^+ secretie inhibeerde door het blokkeren van zowel de apicale V-type H^+ ATPase als de basolaterale K^+ opname via de K^+ kanalen.

Als mogelijke fysiologische functies voor deze antidiuretische factor kunnen er bij *Formica* twee mogelijkheden naar voor geschoven worden. Een eerste mogelijkheid kan te maken hebben met het verdedigingsmechanisme van de mier. De subfamilie der *Formicinae* heeft namelijk geen angel en spuit in geval van verdediging mierenzuur op of naar zijn belager (Löfqvist, 1976). Het mierenzuur zit normaal opgeslagen in een gifklier in het achterlijf. Deze maakt als ze gevuld is meer dan 10% van het lichaamsvolume uit (Graedel and Eisner, 1988). Na lediging zal de mier trachten de klier zo snel mogelijk terug te vullen, een proces dat versneld kan worden als verder verlies van lichaamsvloeistof via de urine onderdrukt wordt door antidiuretische factor(en).

Het onderdrukken van het verlies aan lichaamsvloeistof is ook nodig tijdens de wintermaanden. Het is namelijk zo dat de klasse der werkmieren in de nestbasis overwinteren om de populatie in het voorjaar te activeren (Crane, 1990). Tijdens deze overwintering zijn de overlevingskansen groter als de waterhuishouding goed geregeld is.

Hoofdstuk 2

In dit hoofdstuk werd het signaaltransductiemechanisme van de antidiuretische factor bestudeerd. Onze aandacht ging daarbij vooral uit naar cAMP en wel omwille van de volgende reden. In een vorige studie werd er aangetoond dat het celpermeabele cAMP analoog, chlorophenylthio-cAMP (cpt-cAMP) een dubbel effect heeft op de primaire urineproduktie bij de mier (De Decker, 1993). Bij lage concentraties (10^{-6} - 10^{-5} M) veroorzaakte cpt-cAMP een diuretisch effect, bij hogere concentraties daarentegen werd een antiduretische respons waargenomen. Het kon bijgevolg niet uitgesloten worden dat FopADF de intracellulaire cAMP concentratie zodanig verhoogt, dat er een antidiuretisch effect volgt. Een eventuele betrokkenheid van adenylaat cyclase in de signaaloverdracht van FopADF werd dan ook nagegaan met behulp van de adenylaat cyclase inhibitor SQ22536. Eerst en vooral werd er nagegaan of er bij de mier een adenylaat cyclase aanwezig was dat door SQ22536 kon geinhibeerd worden. Dit laatste bleek bleek het geval, want SQ22536 kon de stimulatie van adenylaat cyclase door forskolin onderdrukken. Forskolin is een adenylaat cyclase activator. Vervolgens werd er nagegaan of het antidiuretisch effect van FopADF onderdrukt kon worden als het werd toegediend in de aanwezigheid van SQ22536. Het antwoord hierop bleek negatief te zijn, of met andere woorden de verkregen respons voor FopADF is onafhankelijk van de adenylaat cyclase activiteit. Schijnbaar werkt FopADF niet via een toename in cAMP. Dit werd nog eens bevestigd door het effect van FopADF op de intracellulaire cAMP concentratie te meten met behulp van een competitieve cAMP assay: FopADF veroorzaakte geen verandering in de cAMP concentratie.

Na uitsluiten van cAMP als tweede boodschapper voor FopADF, werd de stikstof oxide (NO) - cGMP weg bekeken. Deze keuze werd ingegeven door het feit dat CAP_{2b}, het enige volledig gekarakteriseerde insect peptide met een antidiuretisch effect op Malpighische vaatjes, ageert via een toename in intracellulair cGMP; in *Rhodnius* via het membraan geassocieerde guanylaat cyclase (Quinlan et al., 1997) en in *Drosophila*, waar CAP_{2b} een diuretisch effect vertoont, via de cytosol vrije vorm van guanylaat cyclase (Davies et al., 1995). Bijgevolg was het interessant om cGMP als tweede boodschapper bij de mier te bestuderen. De NO-weg werd getest op twee verschillende interferentiepunten. Eerst door het natuurlijk substraat voor NO-synthetase, namelijk L-arginine, toe te dienen en vervolgens door SNAP te gebruiken, een stof die NO vrijstelt. Geen van beide stoffen had een effect op de vloeistofsecretie in geïsoleerde Malpigische vaatjes. Bijgevolg kan er besloten worden dat de NO-cGMP weg bij de mieren niet meetbaar kan geactiveerd worden en/of geen rol speelt in de regeling van de

vloeistofsecretie, waardoor het een slechte kandidaat is voor de signaaltransductie van FopADF.

Finaal werd de bijdrage van calcium bestudeerd. De Malpigische vaatjes van de mier reageren met een toename in vloeistofsecretie wanneer ze worden behandeld met het calcium ionofoor A23187. Een opmerkelijke waarneming werd er gedaan toen de calciumionofoor werd toegevoegd aan buisjes die vooraf behandeld werden met FopADP. Een transiënte, significante toename in vloeistofsecretie werd waargenomen. Dit suggereert dat een toename van intracellulair calcium de primaire urine productie in de Malpigische vaatjes stimuleert via mechanismen die tenminste gedeeltelijk geblokkeerd kunnen worden door FopADP.

Er kon bijgevolg finaal besloten worden dat FopADF werkt op een niveau dat buiten de controle valt van zowel cAMP als calcium. Tot een vergelijkbare conclusie is men gekomen bij het bestuderen van het effect van destruxines op de vloeistofsecretie in *Schistocerca gregaria* (James et al., 1993). Destruxines zijn cyclische peptiden en toxines, die geïsoleerd werden uit de pathogene schimmel *Metarhizium anisopliae* (Kodaira, 1961). Zoals FopADF, hebben ze een antidiuretisch effect op de Malpigische vaatjes van insecten (James et al., 1993).

Hoofdstuk 3

Er werd vroeger reeds aangetoond dat het 15% TFA koppenextract van de mier naast antidiuretische ook diuretische factoren bevat (De Decker et al., 1993) die tot op heden niet volledig gekarakteriseerd konden worden. In dit hoofdstuk wordt beschreven hoe één van deze diuretische factoren uit een 10% TFA kop/thorax extract van 300.000 mieren opgezuiverd en ten dele gekarakteriseerd werd. Het *F. polyctena* diuretische peptide (Fop-DP) werd opgezuiverd in een tweestaps HPLC- opzuiverings protocol, vertrekkende van de 20% acetonitrille (CH_3CN) fractie verkregen na vooropzuivering van het ruwe extract op een C_4 vaste fase extractie kolom. Deze eenvoudige chromatografische opzuivering werd bekomen na voorfractionatie van een klein deel van de 20% CH_3CN fractie op een analystische C_{18} kolom. Het belangrijkste voordeel van deze eerste fractionering (met slechts een kleine hoeveelheid van materiaal) was een toename van de specificiteit in de respons verkregen voor de vloeistofsecretie-experimenten. De chromatografische fractionatie leverde slechts 4 U.V.- absorberende pieken op die manueel werden opgevangen. Slechts in één van deze fracties werd een significante, diuretische respons gevonden. Gebaseerd op dit resultaat kon de rest van het extract op de C_{18} verwerkt worden met collectie van de diuretische fractie op basis van zijn retentietijd en

absorptieprofiel. Deze fractie werd vervolgens verder gezuiverd op een analytische C_4 kolom, wat resulteerde in een opgezuiverd peptide met een moleculaire massa van 7.514 dalton. Bij gebrek aan materiaal werd geen enzymatische hydrolyse uitgevoerd en werd enkel de sequentie van de eerste 25 aminozuren bepaald: VPKYENCVSEVLPAGDRQCVKVTC. Zoeken in databanken gaf geen significante gelijkenis van Fop-DP met andere reeds gekende diuretische peptiden van insecten. Momenteel wordt Fop-DP terug opgezuiverd en aan een behandeling met endopeptidasen onderworpen. De bekomen fragmenten worden van elkaar gescheiden met behulp van HPLC en zullen in de toekomst aminoterminaal gesequeneerd worden.

Er bestaan wel functionele overeenkomsten, namelijk met de myokininen. Voor het opgezuiverde peptide werd het effect op de membraanpotentialen en het signaaltransductiemechanisme onderzocht. Het diuretisch peptide Fop-DP veroorzaakte een depolarizatie van de apicale membraan. Dit was vergelijkbaar met het effect van myokinines op *Drosophila* (O'Donnell et al., 1998) en *Aedes* (Panabecker et al., 1993) Malpigische vaatjes. Ook wat de signaaloverdracht betreft, vertoont Fop-DP gelijkenis met de myokinines. Er werd namelijk aangetoond dat de effecten van Fop-DP, zowel op de vloeistofsecretie als op de membraanpotentiaal, konden worden nagebootst door de calciumionofoor A23187 en door thapsigargin, een inhibitor van het calcium ATPase van het endoplasmatisch reticulum. Bovendien heeft Fop-DP een additief effect bovenop het stimulatorisch effect van cAMP en omgekeerd (Laenen et al., 1998c). Bijgevolg kon besloten worden dat Fop-DP een diuretisch peptide is dat via een toename in cytoplasmatisch calcium werkt, zoals de myokinines.

Hoofdstuk 4

Species van dezelfde insectenfamilie of zelfs van dezelfde insectenorde bezitten proteinen met een laag moleculair gewicht en polypeptiden met vergelijkbare eigenschappen (Gäde, 1989),(Gäde, 1997),(Schmidt et al., 1986). Grotere insecten leveren meer materiaal. Dit kan nuttig zijn in de zoektocht naar neuroendocriene substanties in lichaamsextracten (Holman et al., 1990). Daarom was het interessant om uit te zoeken of er ook endogene diuretische en antidiuretische peptides, vergelijkbaar met diegenen die bij de mier gevonden werden, konden worden aangetoond in de honingbij (*Apis mellifera carnica)* . De honingbij is in grote aantallen beschikbaar en behoort tot dezelfde insectenorde als de bosmier. Bovendien is het een groter insect, waardoor het mogelijk is lichaamsextracten te maken van een kleiner aantal insecten. Hun phylogenetische relatie laat de aanwezigheid vermoeden van vergelijkbare factoren in de weefselextracten van beide species. In dit

hoofdstuk beschrijven we een eerste poging om zulke verwante factoren terug te vinden. Een chromatografische vergelijking werd gemaakt van weefselextract van een klein aantal honingbijen en bosmieren. Er werd aangetoond dat beide insectensoorten vergelijkbare factoren hebben die chromatografisch kunnen worden geïdentificeerd. Het meest opvallend was de aanwezigheid van een antidiuretische factor in het achterlijfextract van de honingbij. Deze factor, nu *A. mellifera* antidiuretische factor (ApmADF) genoemd, heeft ongeveer dezelfde retentietijd als FopADF en heeft vergelijkbare biologische effecten op geïsoleerde Malpigische vaatjes van de mier. Zoals FopADF (Laenen et al., 1998b) heeft ApmADF een reversiebel antidiuretisch effect op de primaire urine productie en veroorzaakt het een depolarizatie van de apicale membraanpotentiaal.
In het koppenextract van de honingbij werd een diuretische factor aangetoond met dezelfde retentietijd als Fop-DP. Deze factor, nu *A. mellifera* diuretische factor (Apm-DF) genoemd, heeft een zwak maar significant, diuretisch effect op Malpigische vaatjes van de bosmier. De lage respons kan te wijten zijn aan het feit dat de mierenbuisjes minder gevoelig zijn voor de bijenfactor, ofwel is Apm-DF geen volwaardige diuretische factor. Of Apm-DF al dan niet functioneert als een diuretische factor in de honingbij zelf, kan enkel onderzocht worden in secretie-experimenten op de Malpigische vaatjes van de honingbij zelf

Hoofdstuk 5
Zoals reeds in de inleiding werd aangehaald, werken bij andere insecten myokinines en "corticotropin-releasing-factor" (CRF)-verwante peptiden samen om de secretiesnelheid te stimuleren tot een maximaal niveau (Coast, 1995),(O'Donnell et al., 1996). Uit alle insectensoorten, die tot op heden bestudeerd werden, konden zowel myotrope als CRF-verwante peptiden opgezuiverd worden. In een voorafgaande studie (De Decker, 1993) werden twee myotrope peptiden geïdentificeerd uit één van de vooropgezuiverde fracties van een 15% TFA koppenextract van de bosmier. Deze twee myotrope peptiden hadden weliswaar geen effect op de primaire urineproduktie, maar tijdens de opzuivering werden er chromatografische fracties gevonden met zowel een myotroop als een diuretisch effect. Het is dus niet uitgesloten dat er ook bij de mier myokinines terug te vinden zijn. Bijgevolg, als de voorgaande situatie van toepassing is voor de mier, moet het mogelijk zijn een CRF geassocieerd peptide bij de mier te identificeren. Tot nu toe werden de meeste CRF-verwante peptiden opgezuiverd uit zure methanol extracten, daarom werd deze extractie procedure vergeleken met de 15% TFA methode die tot hiertoe voor de mier gebruikt werd. De resultaten toonden aan dat beide soorten kop/thorax extract CRF

verwant materiaal bevatten. Gescheiden op een analytische C_8 kolom kon een piek met een stimulerend effect op zowel de urineproductie als de op cAMP-productie aangetoond worden. Stimulatie van vloeistofsecretie en cAMP-productie zijn twee typische kenmerken van CRF verwante peptiden (Coast, 1996). Bovendien had deze piek eenzelfde retentietijd als het *L. migratoria* diuretische hormoon (Lom-DH), het CRF verwante peptide van de Afrikaanse sprinkhaan (Kay et al., 1991), wanneer de chromatografische scheiding onder identieke condities werd uitgevoerd. Het TFA extract bevatte wel meer materiaal en de extractieprocedure was eenvoudiger uit te voeren. Voor het vervolg van de studie werd dan ook besloten om de TFA extractieprocedure te gebruiken. Zoals eerder vermeld verloor het 15% TFA kop/thorax extract van de bosmier een deel van zijn diuretische activiteit na behandeling met aminopeptidase M (De Decker et al., 1993). Dit verlies aan diuretische activiteit suggereerde dat het co-eluerende materiaal peptidergisch is. Een ander bewijs voor de aanwezigheid van een CRF-verwant peptide in het 15% TFA kop / thorax extract van de mier werd bekomen in een immunologische studie. Polyclonale antisera gericht tegen *M. sexta* diuretisch hormoon (Mas-DH) en Lom-DH vertoonden een immunologische respons op de 40% CH_3CN fractie van het mierenextract. Gebaseerd op deze immunoreactiviteit en op het diuretisch effect van Mas-DH op geisoleerde Malpigische vaatjes van de mier (De Decker et al., 1993), wordt er verwacht dat het CRF gelijkende materiaal uit het mierenextract meer overeenkomt met Mas-DH dan met Lom-DH. In de toekomst moet het mogelijk zijn CRF verwante peptide(n) uit de 40% CH_3CN fractie van een 10% TFA kop/thorax extract van de mier op te zuiveren met behulp van zowel de competitieve cAMP binding assay als een ELISA gebaseerd op Mas-DH. Deze opzuivering zou het eerste CRF verwante peptide voor de Hymenoptera familie opleveren.

Hoofdstuk 6

In dit hoofdstuk wordt het resultaat voorgesteld van een eerste poging om CRF-verwante peptiden op te zuiveren bij de mier. We hebben hierbij gebruik gemaakt van het HPLC protocol dat vroeger reeds gebruikt werd voor de opzuivering van CRF-verwante peptiden uit sprinkhanen (Kay et al., 1991), vliegen (Clottens et al., 1994) en kakkerlakken (Kay et al., 1992). Gezien dit werk in het labo van Dr. G.M Coast (Londen) werd uitgevoerd hadden we slecht een beperkt aantal mieren te onzer beschikking. In voorafgaande experimenten werd aangetoond dat het ruwe kop/thorax extract van de mier zowel de cAMP productie als de vloeistofsecretie stimuleert in geïsoleerde Malpigische vaatjes van sprinkhanen. Vandaar dat deze laatsten gebruikt werden voor de screening van de chromatografische fracties. De aanwezigheid van cAMP genererende en diuretische activiteit werd onderzocht. Naast het effect op de intracellulaire cAMP concentratie en de primaire urineproductie werd ook het myotroop effect van de chromatografische fractie op de buisjes van de sprinkhaan bekeken. Er werd voor een aantal insecten aangetoond dat CRF verwante peptiden een myotroop effect hebben op de spier gelegen rond de buisjes van Malpighi (Coast, 1998a). De hoge gevoeligheid van deze assay liet toe de HPLC fracties te screenen zonder voorbehandeling, waardoor de screening naar biologische activiteit tijdens de chromatografische zuiveringsstappen eenvoudiger werd. Kruisreactiviteit van actieve fracties werd bevestigd in secretie-experimenten op Malpigische vaatjes van de mier. Deze benadering leidde tot de opzuivering van het *F. polyctena* cAMP genererend peptide (Fop-cGP). Dit 30-mer peptide met een moleculair gewicht van 2,966 daltons behoorde niet tot de CRF-verwante familie van insectpeptiden en is als dusdanig het tweede diuretisch peptide gevonden in insecten met een cAMP genererende activiteit dat niet tot de CRF-familie behoort. Bij *L. migratoria* had het een myotroop effect op de spiraalvormige spierlaag rond het buisje van Malpighi en verdubbelde het zowel de cAMP productie als de vloeistofsecretiesnelheid. Bij *F. polyctena* werd tot nog toe enkel het effect op de vloeistofsecretiesnelheid getest. Fop-cGP stimuleert de primaire urineproductie, maar de respons van de Malpigische vaatjes van de mier was veel kleiner dan deze van de buisjes van de sprinkhaan. De lage respons zou een indicatie kunnen zijn dat Fop-cGP niet de primaire stimulator is van de vloeistofsecretie in de mieren. Mogelijks ligt zijn belangrijkste fysiologische functie elders of moet er een tweede peptide, bvb Fop-DP, aanwezig zijn om een maximaal effect op de primaire urineproductie te verkrijgen. Er zijn meer experimenten nodig om na te trekken of Fop-cGP en Fop-DP samenwerken en een belangrijke rol spelen in de regeling van de primaire urineproductie van de mier.

Algemeen besluit

De regeling van de waterhomeostase is veel complexer dan aanvankelijk werd gedacht. Zo moet er in eerste instantie een onderscheid gemaakt worden tussen, de regulatie enerzijds op het niveau van de primaire urine produktie ter hoogte van de Malpighische vaatjes en anderzijds op het niveau van de reabsorptie ter hoogte van het ileum / rectum complex (Spring, 1990). Beide systemen worden volledig onafhankelijk van elkaar geregeld. Het aanvankelijk vermoeden dat bij xerische insecten de kinine-achtige factoren als clearance factoren kunnen beschouwd worden, ten gevolge van een stimulatie van de Malpighische vaatjes en verhogen van de reabsorptie ter hoogte van het rectum (Nicolson, 1991), werd recentelijk weerlegd. De verhoogde reabsorptie is geen rechtstreeks effect op het rectum doch wel op het cryptonergisch complex (G.Coast, persoonlijke mededeling).

Voor wat betreft de Malpigische vaatjes betreft, zijn er waarschijnlijk meer factoren betrokken bij de regeling van de secretie dan eerst werd gedacht. Tot op heden had men een zeer eenvoudig beeld voor ogen: twee factoren zouden nodig zijn om de primaire urine productie door de Malpighische vaatjes te regelen (Coast, 1998b). Met name de Corticotropin Releasing Factor (CRF) - verwante peptiden en de myokinines (Maddrell et al., 1993). Met betrekking tot de regulatie blijkt dat er voor de peptiden van deze families, duidelijk sprake is van een cooperatief effect, bij sprinkhanen zijn slechts submaximale concentraties van het LomDH (CRF-verwant peptide) en LomK (myokinine) nodig om een maximaal effect op de secretiesnelheid te verkrijgen (Coast, 1995).

Door dit coöperatief effect zijn de insecten in staat hun secretiesnelheid zeer snel aan te passen zonder grote veranderingen van de peptideconcentraties in het hemolymfe.

Ook bij de mier is een gelijkaardige situatie mogelijk. Zo heeft De Decker *et al.*, 1994 reeds aangetoond dat er bij de bosmier myotrope peptiden terug te vinden zijn. De geïdentificeerde peptiden LomTK I en LomTK II zijn verwant met de tachykinines en hebben geen effect op de primaire urine productie, maar tijdens de opzuivering van deze peptiden werden een aantal chromatografische fracties niet geanalyseerd. In deze fracties werd naast een myotrope ook een diuretische activiteit aangetoond (De Decker, 1993) wat laat vermoeden dat er ook bij de mier myokinines terug te vinden zijn. Het zou dus zeker interessant zijn ook deze fracties verder te bestuderen en misschien FopK te kunnen opzuiveren.

In de huidige studie konden we aantonen dat, bij de mieren CRF-verwant materiaal terug te vinden is in de 40% acetonitrile fractie van een 15% TFA koppen/thorax extract (zie Hfst. 5). Een volledige opzuivering werd nog niet gerealiseerd maar de aanwezigheid van de

factoren kon worden gedetecteerd: de identificatie gebeurde op basis van de biologische activiteit van de chromatografische fracties gelegen rond de elutietijd van Lom-DH, waarbij het referentie peptide gekozen werd op basis van de immunologische respons van het ruwe extract voor polyclonale antisera gericht tegen Lom-DH en Mas-DH.

Toch blijkt de regulatie van de primaire urine productie complexer te zijn. Zo werden bij vliegen recentelijk diuretische peptiden opgezuiverd die struktureel niet bij één van de twee vernoemde families onder te brengen zijn (Spittaels et al., 1996). Wat de activiteit betreft zijn ze te vergelijken met de CRF-verwante peptiden vermits de signaaloverdracht eveneens via cAMP verloopt. Toch vervangen ze de CRF-verwante factoren niet, want bij vliegen werden vroeger reeds CRF-verwante peptiden opgezuiverd (Clottens et al., 1994). Hieruit blijkt dat de regulatie met minstens één factor moet worden uitgebreid. Ook wij zijn er in geslaagd om bij de bosmier een diuretische factor op te zuiveren die als tweede boodschapper cAMP gebruikt en die niet verwant is met de CRF-verwante peptiden (zie Hfst. 6). In hoeverre de factor, FopcGP, een cooperatief gedrag vertoont in de aanwezigheid van CRF-verwante peptiden en/of myokinines blijft nog een open vraag. Het is mogelijk dat deze cAMP genererende factor in de mier de rol van de CRF-verwante peptiden speelt, waarbij de uiteindelijke vrijzetting, functie zal zijn van een externe prikkel. Anderzijds zou het diuretisch effect van de cAMP genererende peptiden slechts een secundair effect kunnen zijn. Te meer daar de opzuivering van deze factor bij de mier werd uitgevoerd op basis van een kruisreactiviteit met de Malpighische vaatjes van de sprinkhaan (zie Hfst. 6) (Laenen et al., 1998a). FopcGP heeft voor de sprinkhaan een zeer duidelijk en potent diuretisch effect dat zelfs gepaard gaat met een myotroop effect op de gladde spier die rondom de Malpighische vaatjes gelegen is. Bij de mier blijft er van deze effecten nog maar zeer weinig over. De secretie wordt maar zeer lichtjes gestimuleerd en van het bijhorende myotrope effect blijft helemaal niets meer overeind. Uitsluitsel over de biologische activiteit van FopcGP voor de mier kan maar verkregen worden op het ogenblik dat we over het synthetisch analoog beschikken. Dit neemt evenwel niet weg dat het effect van de cAMP genererende peptiden misschien enkel een aspecifieke respons van de secretieexperimenten is (Holman and Hayes, 1996).

Moeilijker te plaatsen in dit hele verhaal is dat van het opgezuiverde FopDP. Dit is een relatief groot peptide (7kDa) waarvan we hebben kunnen aantonen dat het calcium als tweede boodschapper gebruikt (zie Hst.3). Bovendien werd een additief effect met cAMP aangetoond waardoor de kenmerken zeer vergelijkbaar zijn met de myokinines. Maar verder gaat de vergelijking niet meer op. FopDP is een cysteine rijk peptide, dat niet te vergelijken is met de andere reeds gekende cysteine rijke peptiden bij insecten (zie Hfst. 3)

(Laenen et al., 1998c). Eventuele overeenkomsten met andere peptiden in het patroon van de cysteine residues kan maar onderzocht worden van zodra de volledige sequentie van het peptide gekend is. Of FopDP naar analogie met FopcGP eventueel de rol van de myokinines overneemt en/of een cooperatief effect vertoont met andere factoren blijft tot op heden een open vraag. Het vastgestelde additief effect van FopDP en cAMP (zie Hst.3) opent in dat opzicht wel de nodige perspectieven.

Het meest opmerkelijke resultaat van deze studie is ongetwijfeld de aanwezigheid van een antidiuretische factor in hemolymfe extracten van de bosmier. Een vermoeden dat de mier dergelijke factoren bezat was afkomstig van het feit dat de basale secretiesnelheid van de Malpighische vaatjes sterk kon verschillen en soms zelfs onmeetbaar klein was (De Decker, 1993). Het is tot dusver het derde voorbeeld waarbij er een rechtstreeks antidiuretisch effect kon worden aangetoond voor het toedienen van een exo- of endogene factor aan de Malpigische vaatjes. Tot voor kort ging men er van uit dat antidiuretische effecten enkel gerealiseerd werden door een verhoogde reabsorptie ter hoogte van het ileum/rectum complex. De Malpigische vaatjes zouden steeds voor een basale urineproduktie zorgen die onder invloed van een aantal factoren kon worden gestimuleerd (Spring, 1990). De uiteindelijke regulatie van de hoeveelheid water die uitgescheiden wordt, gebeurde volgens dit model dus ter hoogte van het ileum/rectum complex. Dit blijkt niet helemaal te kloppen, schijnbaar is het mogelijk om het waterverlies te beperken reeds ter hoogte van de Malpigische vaatjes door het onderdrukken van de primaire urine produktie (Quinlan et al., 1997). Het bestaan van antidiuretische factoren met een rechtstreeks effect op de Malpigische vaatjes, kan begrepen worden in het licht van een aantal reeds gekende fysiologische karakteristieken. Bij de mier zouden ze een rol kunnen spelen in de waterhuishouding na ledigen van de mierenzuurklier bij de verdediging of tijdens de overwintering (zie Hfst.1). Bij bloedzuigende insecten ziet men dat de secretiesnelheid tijdens het bloedzuigen zeer snel toeneemt onder invloed van een zogenaamd natriuretic peptide (Wheelock et al., 1988), maar na de maaltijd wordt de secretie ook snel opnieuw onderdrukt tot op controle niveau. Tot op heden ging men er van uit dat deze afname in secretiesnelheid te wijten was aan de enzymatische afbraak van het natriuretic peptide waardoor de secretie niet onderhouden werd (Wigglesworth, 1931). Het bestaan van de antidiuretische factoren laat toe de afname te verklaren door het uitschakelen van de primaire urine produktie in de Malpigische vaatjes onder invloed van gesecreteerde antidiuretische factoren (Quinlan et al., 1997),(Petzel and Conlon, 1991).

Struktureel hebben we over de beschreven factoren nog maar weinig gegevens. Enkel voor CAP_{2b} en destruxin, twee exogene factoren met een antidiuretisch effect op de

primaire urine produktie, is de struktuur gekend. Deze factoren worden gekenmerkt door een goede bescherming tegen endopeptidase activiteit (Huesmann et al., 1995),(Kodaira, 1961). Nog moeilijker wordt het als we de tweede boodschappers gaan bekijken. Voor CAP_{2b} heeft men eenduidig kunnen aantonen dat het antidiuretisch effect gepaard gaat met een toename in cGMP (Quinlan et al., 1997). Voor FopADF en destruxins is nog niet duidelijk wat de tweede boodschapper is, wat er op kan wijzen dat deze factoren een aspecifiek toxisch effect induceren op de transportmechanismen betrokken in de primaire urine produktie door de Malpigische vaatjes. Duidelijke aanwijzingen hieromtrent hebben we niet, maar voor de mier konden we aantonen dat het effect volledig reversiebel is, zelfs zodanig dat bij aanwezigheid van cpt-cAMP een stimulatie optreedt bij uitwassen van de antidiuretische factor, wat op een behoud in celvitaliteit wijst (zie Hfst. 2) (Laenen et al., 1998b). Gelijkaardige effecten werden waargenomen voor de Malpigische vaatjes van de meelworm *T. molitor*: FopADF inhibeert de vloeistofsecretie en cAMP stimuleert na wegwassen van FopADF. Bovendien kon FopADF aangetoond worden in het hemolymfe extract van niet secreterende mieren wat er op wijst dat deze factor wordt vrijgesteld onder invloed van nog nader te definiëren factoren (zie Hfst.1). Een volledige karakterisatie van de factor zal echter nodig zijn om de precieze werking te kunnen achterhalen. Na bereiden van synthetische analogen van de opgezuiverde peptiden openen er zich een aantal toekomstperspectieven. Zo wordt het mogelijk om polyclonale antilichamen aan te maken waarna men met behulp van immunocytochemie kan nagaan waar de betrokken factoren gesynthetiseerd worden en waar ze worden vrijgesteld. Zo hebben we tot op heden geen enkel bewijs om aan te tonen dat het in deze studie over neuroendocriene factoren gaat. Enkel voor de diuretische factoren die opgezuiverd werden uit kopextracten, kunnen we stellen en dat de synthese en/of release site in de kop gelegen is.

Samenvattend hopen we met ons werk een bijdrage geleverd te hebben en te kunnen besluiten dat de regeling van de waterhuishouding ter hoogte van de Malpighische vaatjes veel complexer is dan aanvankelijk werd gedacht. Zo vonden we aanwijzingen dat er naast de CRF-verwante peptiden en de myokinines ook nog andere factoren een rol spelen. Opmerkelijk is dat deze andere factoren vermoedelijk van dezelfde boodschappers gebruik maken. Zo zouden de cAMP genererende peptiden, de rol van de CRF-verwante peptiden en de grote cysteine rijke peptiden zoals FopDP de rol van de myokinines kunnen overnemen in de mier. Daarenboven zijn er naast duiretische ook antidiuretische factoren betrokken in de regeling van de primaire urine produktie ter hoogte van de Malpigische vaatjes.

SUMMARY

The Malpighian tubules of the forest ant, *F. polyctena*, have been studied for many years now by the research group physiology of the Limburgs Universitair Centrum (Diepenbeek, Belgium). In insects these tubules are responsible for the primary urine production. The latter is achieved via secretion, driven by the active transport of KCl from hemolymph to lumen, and passive diffusion of substances. Initially the transport mechanisms involved in this active transport have been investigated. In a subsequent phase the factors involved in the regulation of this active transport were investigated, i.e. the presence of endogenous factors with a direct effect on the primary urine production by the Malpighian tubules was investigated. Both diuretic and antidiuretic factors were found to be present in body extracts of the ants. Some of these factors, i.e. a diuretic peptide (Fop-DP I) and two myotropic peptides (Fop-TK I en Fop-TK II), could be purified. In the present study we tried to further complete this picture. Different factors, both diuretic (Fop-DP and Fop-cGP) and antidiuretic (FopADF), were shown to be present in body extracts of the ant and some could be identified.

The antidiuretic factor, purified from an abdomen extract, was shown to block the transportmechanisms involved in the active transport of K^+ through the cells of the Malpighian tubules (see chapter 1).

The signal transduction pathway used by FopADF could not be clarified. The involvement of adenylate cyclase and / or an increase in intracellular cAMP could clearly be excluded (see chapter 2). A cross-reactivity of *Tenebrio molitor* Malpighian tubules towards FopADF could be demonstrated by Dr. S. Nicolson (see Note after chapter 1) and a similar antidiuretic factor was shown to be present in an abdomen extract of the honeybee, *Apis mellifer* (see chapter 4).

The diuretic factors, both purified from head / thorax extracts, were shown to be peptides. For Fop-DP approximately 35% of the amino acid sequence could be determined. Fop-DP acted via an increase in intracellular calcium (see Chapter 3). A Fop-DP related factor was also demonstrated to be present in a head extract of the honeybee (see chapter 4).

The second diuretic factor, Fop-cGP, was fully characterized. It is a peptide of 30 amino acids which exerts its diuretic effect in Locust, via an increase in cAMP (see Chapter 6). The purification of Fop-cGP followed a preliminary study trying to establish the presence of CRF-related peptides in the forest ant. The latter were shown to be present in a head / thorax extract of the ant by means of chromatography and an ELISA based on polyclonal antisera to CRF-related peptides from other insects (see Chapter 5). Fop-cGP had the

activity of a CRF-like factor but not the amino acid composition. Another factor having a CRF-like structure may be present but still awaits further purification and characterization. Below we tried to give a brief overview of the relevant results for each chapter. A table, added as bookmarker, summarizes the results and is intended as a reader guide. The factors involved in the endocrine regulation of the primary urine production of the forest ant is compared to the situation known for other insects.

Chapter one

As mentioned in the introduction, antidiuretic factor(s) were shown to be present in head extracts of the ant (De Decker et al., 1994). In the current study it was demonstrated that antidiuretic factor(s) were also present in the ant hemolymph. A hemolymph extract, made from insects with non-secreting Malpighian tubules, had an antidiuretic effect on the primary urine production in ants with secreting tubules. Since the collection of hemolymph fluid from the abdomen was time consuming, a 15% trifluoroacetic acid extract was made of whole abdomina of 150,000 ants. This abdomen extract also had an inhibitory effect in the fluid secretion assay and was used in an effort to identify for the first time a factor responsible for the antidiuretic response.

A chromatographically pure antidiuretic factor was obtained and was named *Formica polyctena* antidiuretic factor (FopADF) according to the insect peptide nomenclature proposed by Raina and Gäde, 1988 (Raina and Gäde, 1988). The molecular characterization failed. This could be due to the fact that we were dealing with a cyclic peptide, as suggested in the Discussion. Besides the reversible antidiuretic effect in the fluid secretion assay, the effect of FopADF on the membrane potentials was investigated. We found that FopADF depolarized both the apical and the basolateral membrane potentials, a response which was reversible upon washout. A similar response had previously been reported for the combined effect of the metabolic inhibitor dinitrophenol (DNP) and barium, a selective blocker of potassium channels (Leyssens et al., 1993), on single isolated Malpighian tubules of the ant. This suggested that FopADF inhibited the active potassium uptake via K^+ secretion by blocking both the active V-type H^+ ATPase in the apical membrane and the potassium channels in the basolateral membrane. Since an inhibitory effect on the primary urine formation of secreting tubules was present in a hemolymph extract collected from stressed animals, the physiological role of the antidiuretic factor may be prevention of further fluid loss after emptying of the poison gland in stress conditions.

Chapter two

In this Chapter we tried to find out which signal transduction pathway was used by the antidiuretic factor. A first candidate was an increase in cAMP, especially since De Decker *et al.* 1993 (De Decker, 1993), reported a dual effect for cAMP in ant tubules. At low (10^{-6} - 10^{-5} M) concentrations the cell permeable cpt-cAMP had a diuretic effect, at higher concentrations an antidiuretic response was seen. So, possibly FopADF used cAMP as second messenger. This was checked both indirectly via adenylate cyclase activators and inhibitors and directly through assessment of the intracellular cAMP concentrations. The results showed that FopADF did not use the adenylate cyclase - cAMP pathway.

Up till now CAP_{2b} is the only insect peptide (from *M. Sexta*), with an antidiuretic effect on insect Malpighian tubules (of *Rhodnius* or *Drosophila*), for which the signal transduction mechanism is known. In the insect species studied, it exerted its effect via an increase in cGMP; in *Rhodnius* via the membrane associated guanylate cyclase (Quinlan et al., 1997) and in *Drosphila*, where it has a diuretic effect, via the soluble form of guanylate cyclase (Davies et al., 1995). It was consequently of interest to investigate the role of cGMP as a second messenger in the ant Malpighian tubules.

The NO-pathway has been tested at two different points of interference. First of all by offering the natural substrate for NO-synthetase, L-arginine, to the tubules and secondly by applying SNAP, a NO-releasing agent. None of the agents had any effect on the fluid secretion in single isolated Malpighian tubules. It could consequently be concluded that the NO-cGMP pathway could not be shown to affect fluid secretion in the ant tubules and that it is an unlikely candidate for the signal transduction pathway of FopADF.

Finally the contribution of calcium has been investigated. The ant Malpighian tubules responded with an increase in fluid secretion when they were challenged with the calcium ionophore A23187 or the intracellular calcium mobilizing drug, thapsigargin. When the calcium ionophore was added to tubules which were previously treated with FopADF, a transient, significant stimulation of the fluid secretion rate was seen. This suggested that an increase in intracellular calcium stimulated the primary urine production in ant tubules via mechanisms which are at least partially blocked by FopADF. It could consequently be concluded that FopADF acted at a level beyond the control of either cAMP, cGMP or calcium. A similar conclusion was reached for the inhibition by cyclic peptide toxins, destruxins, of fluid secretion in *Schistocerca gregaria* (James et al., 1993).

Chapter three

In a previous study it was shown that a crude 15% TFA head extract of the ant contained diuretic peptides (De Decker et al., 1993). In the present study we identified one of these diuretic factors in a 10% TFA head / thorax extract of 300,000 ants. The *Formica polyctena* diuretic factor (Fop-DP) was purified in a two step HPLC-purification protocol from the 20% CH_3CN fraction, obtained after prepurification of the crude extract over reversed-phase C_4 cartridges. First of all a small part of the 20% CH_3CN fraction was chromatographically purified and studied in detail. A major advantage of using a small amount of material is avoiding aspecific effects. Screening the fractions for their effect on fluid secretion for instance will be more specific (1993). Only four U.V.-absorbing peak fractions were manually collected and in one of them a potent, significant diuretic effect was found. This result allowed us to upscale the loading of the rest of the extract on the same analytical C_{18}-column and to collect the diuretic fraction based on its retention time and absorption profile. This fraction was further purified on an analytical C_4-column and resulted in a purified peptide with a molecular mass of 7,514 daltons. Due to lack of material, no enzymatic digestion could be performed and the sequence of only the first 25 amino acids could be determined : VPKYENCVSEVLPAGDRQRCVKVTC. A computer search of sequence data banks did not reveal any significant similarity of Fop-DP with other known insect diuretic peptides. In current experiments Fop-DP is being repurified and is submitted to treatment with endopeptidases. The fragments obtained are separated from one another by means of HPLC and will be aminoterminally sequenced.

Although the molecular structure of Fop-DP is different from the myokinins, its physiological activity was very similar. The response of the membrane potentials to Fop-DP was investigated and showed a depolarization of the apical membrane. This was comparable to the effect of the myokinins on *Drosophila* (O'Donnell et al., 1998) and *Aedes* (Panabecker et al., 1993) tubules. Fop-DP also paralleled the biological effect of the myokinins with respect to the signal transduction pathway. As mentioned in the introduction, the myokinins exert their diuretic effect via an increase in intracellular calcium. The effects of Fop-DP, both on fluid secretion and on membrane potentials, was mimicked by the calcium ionophore A23187 or by thapsigargin, an inhibitor of the endoplasmic reticulum calcium ATPase. Moreover, Fop-DP had an additive effect on top of the stimulatory effect of cAMP and vice versa. It could consequently be concluded that Fop-DP is a diuretic peptide, that acts via an increase in intracellular calcium.

Chapter four

Species from the same insect family or even the same insect order have been shown to possess low molecular weight proteins and polypeptides with similar properties (Gäde, 1989),(Gäde, 1997),(Schmidt et al., 1986). Larger insects provide more material in the search for neuroendocrine substances in body extracts (Holman et al., 1990). It seemed therefore interesting to find out whether endogenous diuretic and antidiuretic peptides, similar to those found in the ant, were present in the honeybee (*Apis mellifera carnica*). The honeybee was available in large numbers and it belonged to the same insect order as the forest ant. Furthermore it is a larger insect and therefore the same amount of peptide can be obtained from a smaller number of insects. Due to their phylogenetic relation the presence of similar factors in the tissue extracts of both insect species is expected.

In this chapter we presented the preliminary results of a chromatographic comparison of tissue extracts of a limited number of animals, either the honeybee or the forest ant. It was found that both insect species contained similar factors which could be chromatograpically identified. Most remarkable was the presence of an antidiuretic factor in an abdomen extract of the honeybee. This factor, hereafter named *A. mellifera* antidiuretic factor (ApmADF), had almost the same retention time as FopADF and had similar biological effects on single isolated Malpighian tubules of the ant (Laenen et al., 1998b). ApmADF had a reversible antidiuretic effect on the primary urine production and depolarized the apical membrane potential of the ant's Malpighian tubules in a reversible way.

Furthermore, in a head / thorax extract of the bee a diuretic factor was found, which had the same retention time as Fop-DP. This factor, hereafter named *A. mellifera* diuretic factor (Apm-DF), had a small diuretic effect on ant tubules. The low response may be due to the fact that the ant tubules are less sensitive to Apm-DF. Whether Apm-DF functions as a diuretic factor in the bee can only be investigated by testing it on single isolated Malpighian tubules of the honeybee.

Chapter five

As mentioned in the introduction, in many insects, myokinins and corticotropin-releasing-factor (CRF) - related peptides act synergetically to stimulate the fluid secretion rate to a maximal level (Coast, 1995),(O'Donnell et al., 1996). Besides, up till now both myotropic peptides and CRF-related peptides were purified from all the insect species studied so far. In a previous study (De Decker, 1993), myotropic factors with a diuretic effect were shown to be present in a 15% TFA head extract of the forest ant. If these factors are myokinins and if the above observations apply for the ant, then it should be possible to

identify a CRF-related ant peptide. Up till now, most CRF-related peptides were purified from acidic methanol extracts, so we compared this extraction procedure to the 15% TFA extraction used up to now for the ant. The results suggested that both procedures yielded head / thorax extracts containing CRF-related material. Separated on an analytical C8-column the presence of one peak which stimulated both the primary urine production and cAMP production by isolated Malpighian tubules was revealed. Stimulation of fluid secretion and cAMP production are two typical characteristics of CRF-related peptides (Coast, 1996). Furthermore, this peak had the same retention time as *L. migratoria* diuretic hormone (Lom-DH), the CRF-related peptide of the migratory locust (Kay et al., 1991), when chromatographed under identical conditions. The TFA extract contained more material and the extraction procedure was easier to perform than the methanol extraction, it was consequently used for the rest of the present study. As reported previously, the diuretic activity of an ant 15% TFA head / thorax extract is lost after treatment with aminopeptidase M (De Decker et al., 1993), which suggested that the co-eluting material is peptidergic. Further proof for the presence of CRF-related peptides in the 15% TFA head / thorax extract of the ant was obtained from the positive immunoreactive response to polyclonal antisera against both *M. sexta* diuretic hormone (Mas-DH) and Lom-DH to the 40% CH_3CN fraction. Based on this immunoreactivity and on the diuretic effect of Mas-DH on single isolated Malpighian tubules of the ant (De Decker et al., 1993), it is expected that the CRF-like material present in the ant extract is more related to Mas-DH than to Lom-DH. In future experiments it should be possible to purify the CRF-related ant peptide from the 40% CH_3CN fraction of a 10% TFA head / thorax extract, using a combination of a competitive cAMP binding assay and a Mas-DH based ELISA. This purification could provide the first CRF-related peptide in a species of the Hymenoptera family.

Chapter six

In this final chapter we report about a first attempt to purify the CRF-related ant peptide, using the HPLC-protocol used previously for the purification of CRF-related peptides from locusts (Kay et al., 1991), flies (Clottens et al., 1994) and cockroaches (Kay et al., 1992). Since this work was performed in the laboratory of Dr. G.M. Coast (London), only a limited number of ants were available and cross-reactivity with other species was used to screen the chromatographic fractions. In preliminary experiments it was shown that the crude ant head / thorac extract stimulated both the cAMP production and the fluid secretion rate in single isolated Malpighian tubules of the locust. So, the latter were used to screen the chromatographic fractions for the presence of cAMP generating and diuretic activity.

Besides the effects on intracellular cAMP and primary urine production, we also monitored the myotropic effect of the chromatographic fractions on locust tubules. Myotropic effects of CRF-related peptides on the spiral muscle layer around the Malpighian tubule have been observed for a number of insects (Coast, 1998a). The high sensitivity of this assay allowed to screen the HPLC-fractions directly, i.e. at very low concentrations so that no pretreatment was needed. This facilitated the search for biological activity during the purification process. Cross-reactivity of the positive fractions was confirmed in secretion assays on ant tubules. This approach led to the purification of *Formica polyctena* cAMP generating peptide (Fop-cGP). This 30-mer peptide with a molecular mass of 2,966 daltons did not belong to the CRF-related family of insect diuretic peptide as far as structure was concerned. In *L. migratoria* it had a myotropic effect on the spiral muscle layer around the Malpighian tubules and it doubled both the cAMP-production and the fluid secretion rate. In *F. polyctena* only its effect on the fluid secretion rate has been tested so far. Fop-cGP stimulated the primary urine production but the response was much lower than that *Locust* tubules The limited response on the primary urine production might indicate that Fop-cGP is not the prime stimulator of fluid secretion in worker ants. Either its most important physiological function lies elsewhere or a second peptide for instance Fop-cGP must be present to obtain a maximal effect on the primary urine production. Further experiments are needed to investigate whether Fop-cGP and Fop-DP do cooperate and play a primary role in the control of the ant primary urine production.

General conclusion

The waterhomeostasis in insects is much complexer than generally accepted. A first differentiation has to be made between the regulation at the level of the primary urine production by the Malpighian tubules and the regulation at the level of the reabsorption by the ileum/rectum complex (Spring, 1990). Both systems are regulated independently of one another. The initial presumption that the kinin-like factors in xeric insects could be considered as clearance factors, since they stimulated both Malpighian tubules fluid secretion and the water reabsorption in the rectum, was refuted recently. The increase in water reabsorption was not due to a direct effect on the rectum (Nicolson, 1991), but was due to an effect on the cryptonergic complex (G. M. Coast, personal communications).

The regulation of secretion of the Malpighian tubules is also more complex than originally thought. The model describing the regulation of the primary urine production was quite simple: only two factors were thought to regulate the primary urine production by the Malpighian tubules (Coast, 1998b). Namely the Corticotropin Releasing Factor (CRF) -

like peptides and the myokinins (Maddrell et al., 1993). Peptides of these families were shown to have cooperative effects, in locusts only submaximal concentrations of both LomDH (CRF-like peptide) and LomK (myokinin) are needed to get a maximal effect on the fluid secretion rate (Coast, 1995).

By this cooperative effect the insects are capable to adjust the secretion rate swiftly without major changes in the peptide concentrations in the hemolymph. A similar situation may exist in the ant. De Decker *et al.*,1994 demonstrated that myotropic peptides were present in the forest ant. The identified peptides LomTK I and LomTK II were related with the tachykinins and have no effect on the primary urine production, but during the purification a number of chromatographic fractions have not been analysed. These fractions contained both a myotropic and a diuretic activity (De Decker, 1993) which suggest that the forest ant might possess myokinins. So further analysis of these chromatographic fractions wth a possible purification of Fop K would be interesting for future research.

In the present study it was shown that the ants possess CRF-like material in the 40% acetonitrile fraction of a 15% TFA head/thoraces extract (see Chapter 5). A complete purification and characterization is needed, but the presence of the factors could be detected: the identification was based on the biological activity of the chromatographic fractions eluting around the elution time of LomDH. LomDH was chosen as a reference based on the immunological response of the crude extract to polyclonal antisera against Lom-DH and Mas-DH.

Nevertheless, as stated above, the regulation of the primary urine production seems to be more complicated. Only recently two diuretic peptides which could not be classified as a myokinin or a CRF-like peptide, have been purified from flies (Spittaels et al., 1996). Their biological activity could be compared with that of the CRF-like peptides since they also use cAMP as a second messenger. But apparantly they do not replace the CRF-like peptides since in flies CRF-like peptides have also been identified (Clottens et al., 1994). Soat least one additional factor seems to be involved in the regulation of the primary urine production. An idea supported by the fact that in head extracts of the ant a cAMP generating peptide was purified which was not related to the CRF-like peptides (see Chapter 6). Whether this factor, FopcGP, has a cooperative effect in the presence of CRF-like peptides and/or myokinins remains an open question. It might be that in the ant the cAMP generating factors take over the role of the CRF-like peptides and that they are released , CRF-like or cAMP generating, as a function of external stimuli. On the other hand the diuretic effect of the cAMP generating peptides might be a secundary effect, the more so, while the purification of the factor of the ant was based on cross-reactivity with

the Malpighian tubules of the african locust (Laenen et al., 1998a). In locusts FopcGP has a clear and potent diuretic effect which is accompanied by a myotropic effect on the smooth muscle of the Malpighian tubules. In the ant only a minor diuretic effect is present. To investigate the biological activity in the ant we will need the synthetic analogue.

Another completely novel peptide is the purified diuretic peptide FopDP. FopDP is a large (7 kDa) cysteine rich peptide, shown to use calcium as second messenger (see Chapter 3). Moreover it has an additive effect with cAMP and as a consequence its functional characteristics are comparable to those of the myokinins (Laenen et al., 1998c). Whether the cysteine pattern is comparable to the pattern of other cysteine peptides requires the full sequence of the peptide. Also whether FopDP fulfills the role of the myokinins and whether it is cooperative with the other factors still remains an open question.

The most remarkable result of this study is the presence of an antidiuretic factor in hemolymph extracts of the forest ant. It is so far the third example of a direct antidiuretic effect by exo- or endogenous factors on the Malpighian tubules. Until recently it was generally accepted that antidiuresis was realised by an increased water reabsorption at the ileum/rectum complex. The Malpighian tubules were responsible for a basal urine production which could be stimulated by a number of factors (Spring, 1990). The final regulation of the amount of water lost was done at the ileum/rectum complex. This is not entirely true, apparantly water loss can be limited by suppressing the primary urine production in the Malpighian tubules (Quinlan et al., 1997). The presence of antidiuretic factors with a direct effect on the Malpighian tubules can be understood in the light of a number of physiological characteristics of the animals. In the ants they might have a role in the water balance after emptying the formic acid glance or during the survival during winter (see Chapter 1). In hemathophagous insects the fluid secretion is stimulated by the natriuretic factor during a blood meal (Wheelock et al., 1988). Fluid secretion is strongly stimulated during a meal, but recovers very quickly after the meal. Up till now it was generally accepted that this decrease was due to the enzymatic degradation of the natriuretic peptide (Wigglesworth, 1931), but if antidiuretic factors exist the decrease might also be due to the subsequent release of such factors (Quinlan et al., 1997),(Petzel and Conlon, 1991). We have no clear structural information about these antidiuretic factors. Also the signal transduction mechanism is not known. CAP_{2b} and destruxins, exogenous factors with an antidiuretic effect on the primary urine production, are the only two factors of which the structure is known (Huesmann et al., 1995),(Kodaira, 1961). For CAP_{2b} this antidiuretic effect was accompanied by an increase in cGMP (Quinlan et al., 1997). For FopADF and destruxins no signal transduction pathway could be characterized. This

might indicate that their antidiuretic effect is due to toxic aspecific effects induced on the transport mechanisms involved in the primary urine production by the Malpighian tubules. But, in the ants we demonstrated that the effect was fully reversible and in the presence of cpt-cAMP a stimulation was observed upon washout of the antidiuretic factor. The latter suggested that the cells are still viable (Laenen et al., 1998b). Similar effects were observed for the response of *T. molitor* Malpighian tubules to FopADF. Furthermore FopADF was shown to be present in hemolymph extracts of non-secreting ants which suggests that the latter is released into the hemolymph of these ants. A full characterization of FopADF will be necessary to identify its mode of action.
When synthetic analogues of the purified peptides become available it will be possible to investigate the site of synthesis and release in the insect by means of immunocytochemistry. Up till now we have no conclusive arguments as to the neuroendocrine nature of the isolated factors. Only the diuretic factors which have been purified from head extracts are expected to be synthesised and/or released in the ant`s brain.

In summary, our work suggests that the regulation of the water homeostasis at the level of insect Malpighian tubules is much more complicated than hitherto accepted. Besides the CRF-like peptides and the myokinins, other factors are probably involved. Remarkable is that these other factors use the same second messengers. It might be that in the ant the cAMP generating peptides take over the role of the CRF-like peptides and that the large cysteine rich peptides such as FopDP take over the role of the myokinins. In addition antidiuretic factors might be involved in the primary urine production by the insect Malpighian tubules.

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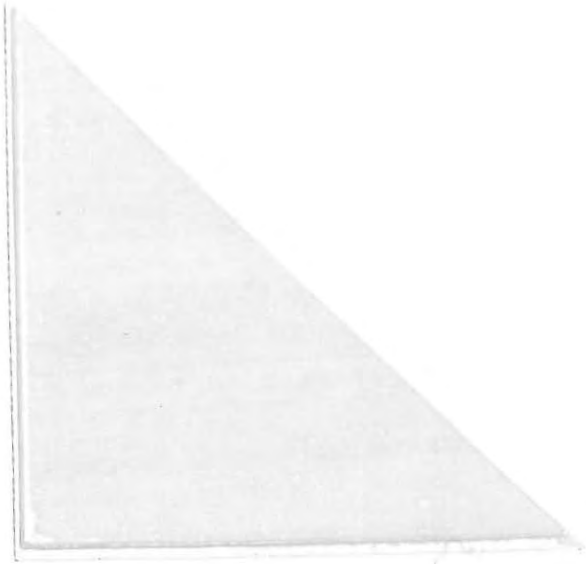

Overview of factors active in the endocrine regulation of primary urine production in other insects as compared to the ant.

Situation in other insects	Antidiuretic		Diuretic		
Name	CAP_{2b} (*Rhodnius*)	Destruxin (*Schistocerca*)	cGP (*Musca*)	CRF -like (Species*)	Myokinin (Species#)
Origin	Exogenous	Exogenous	Endogenous	Endogenous	Endogenous
Structure	NH_2-C terminal blocked	Cyclic peptide	40-50 AA 40-50% sequence homology	30-40 AA core sequence	5-10 AA
Second messenger	cGMP	Acts beyond Ca^{2+} and cAMP	cAMP	cAMP	Calcium
Co-operative with				Myokinin	CRF like
References	(Coast et al., 1993)	(James et al., 1993)	(Spittaels et al., 1996)	(Coast, 1996)	(Gäde et al., 1997)

Situation in the ant	Antidiuretic	Diuretic			
Name	Fop ADF	Fop cGP	CRF -like	FopTK I-II §	FopDP
Origin of prepurified fraction§	Endogenous 60% abdomen	Endogenous 40% head/thorax	Endogenous 40% head/thorax	Endogenous 40% head/thorax	Endogenous 20% head/thorax
Structure	Possibly a cyclic peptide	30 AA	Immunologically and chromatographically identified	9 AA	70 AA
Second messenger	Acts beyond Ca^{2+} and cAMP	cAMP	cAMP	N.D.A.	Calcium
Co-operative with	N.D.A.	N.D.A.	N.D.A.	N.D.A.	cAMP
References	(Laenen et al., 1998)	(Laenen et al., 1998)		(De Decker et al., 1993)	(Laenen, 1998)
Description	Chapters 1 & 2	Chapter 6	Chapter 5		Chapter 3

* *Manduca sexta, Acheta domesticus, Locusta migratoria, Musca domestica, Stomoxis calcitrans, Leucophea madeara*

\# *Manduca sexta, Acheta domesticus, Locusta migratoria, Culex salinarius, Aedes aegypti, Heliothis zea*

§ for the ant myotropic peptides, it has not been determined yet whether they are diuretic or not.

§ the % refers to the acetonitrile fractions obtained after prepurification of the crude extracts over reversed-phase cartridges.

N.D.A. : No Data Available