CALCIUM (Ca^{2+}) AS THE SIGNAL TRANSDUCING ELEMENT IN THE ACTION OF INSECT NEUROPEPTIDE HORMONES IN *IPHITA LIMBATA* STÅL (PYRRHOCORIDAE: HETEROPTERA)

Thesis
submitted to the University of Calicut
in partial fulfilment of the requirements for the Degree of
DOCTOR OF PHILOSOPHY
in Zoology

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APRIL - 2008

ABBREVIATIONS

Å	Angstrom
AC	Adenyl/adenylyl/adenylate cyclase
ADP	Antidiuretic peptide(s)
AKH	Adipokinetic hormone(s)
APRP	AKH precursor related peptide(s)
ATP	Adenosine triphosphate
BAPTA-AM	1,2-bis(aminophenoxy)ethane-N,N,N',N'-tetraacetic acid, acetoxy methyl ester
Bld-HrTH	*Blaberus discoidalis* hypertrehalosaemic hormone
BSA	Bovine serum albumin
^{o}C	degree Celsius
CA	Corpus allatum/Corpora allata
Ca^{2+}	Calcium ion(s)
Ca^{2+}_{o}	Extracellular calcium
Ca^{2+}_{i}	Intracellular calcium
$[Ca^{2+}]_{i}$	Intracellular calcium concentration/ cytosolic concentration of Ca^{2+}
cADPR	Cyclic adenosine diphosphate-ribose
CAH	Cardioacceleratory hormone(s)
cAMP	Cyclic adenosine monophosphate
CAP	Cardioacceleratory peptide(s)
CC	Corpus cardiacum/Corpora cardiaca
CCAP	Crustacean cardioactive peptide
Cd^{2+}	Cadmium ion(s)
cGMP	Cyclic guanosine monophosphate
Co^{2+}	Cobalt ion(s)
CRF-related DH	Corticotropin-releasing factor-related diuretic hormone
DAG	Diacylglycerol(s)
DMSO	Dimethyl sulphoxide
EGTA	Ethylene glycol-0,0'-bis (2-aminoethyl) N,N,N'N'-tetraacetic acid
EH	Eclosion hormone
ER	Endoplasmic reticulum
g	gram(s)
gpe	Gland pair equivalent
h	hours
HDLP	High density lipoprotein
HoTH	Hypotrehalosaemic hormone
HTH/HrTH	Hypertrehalosaemic hormone
i.d.	Inner diameter
IP_3	Inositol 1,4,5-trisphosphate

IP_3R	IP_3-sensitive receptor
ITP	Ion transport peptide
JH	Juvenile hormone
kDa	kilo Daltons
La^{3+}	Lanthanum ion(s)
LDLP	Low density lipoprotein
Lom-AKH-I	*Locusta migratoria* adipokinetic hormone-I
mg	milligram
Mg^{2+}	Magnesium ions
min	minutes
ml	millilitre
mm	millimetre
mM	millimolar
μg	microgram
μl	microlitre
μM	micromolar
μm	micrometre
μmol	micromole
MT	Malpighian tubule(s)
NCC	Nervi corporis cardiaci
nm	nanometre
NSC	Neurosecretory cell(s)
PBAN	Pheromone biosynthesis activating hormone(s)
Pea-HTH	*Periplaneta americana* hypertrehalosaemic hormone
PG	Prothoracic gland(s)
PLA_2	Phospholipase A_2
pmol	picomole
PTTH	Prothoracicotropic hormone(s)
RP-HPLC	Reverse phase- high performance liquid chromatography
RPCH	Red pigment concentrating hormone
rpm	Revolution per minute
RyR	Rynodine sensitive receptor
TAG	Triacylglycerol(s)
TFA	Trifluoroacetic acid
UV	Ultraviolet

STATISTICAL NOTATIONS

ANOVA	Analysis of variance
df	Degree of Freedom
F	F-ratio
P	Probability
SEMs	Standard Error of Means

Acknowledgements

I express my profound sense of gratitude and indebtedness to my Supervising Teacher and Guide, Dr. M. Gokuldas, Professor and Head of the Department of Zoology, University of Calicut, who put his enormous store of experience and knowledge at my disposal.

I express my sincere thanks to the Professors and former Heads of the Department of Zoology, Dr. V.S. Krishnan Nair and Dr. K.P. Janardanan, for providing all the facilities in the Department especially in the Insect Physiology and Biochemistry laboratory.

I also acknowledge, Dr. Manoharan, Reader in Statistics, University of Calicut for his assistance in analyzing the data statistically.

I express my sincere gratitude to my colleagues in the Insect Physiology and Biochemistry Laboratory especially Ajaykumar. A.P., Shalini. C.S. and Binitha. V.S., for the help rendered during the tenure of my work. I also remember the help rendered by Nithya. V., Vidhya. M., Mashoor. K., Sreejith. K. and Smitha. V.

The financial assistance provided by the University Grants Commission, New Delhi, is gratefully acknowledged.

Co-operation from Principal and Management Committee of MES Mampad College is also acknowledged.

I am grateful to M/s Bina Photostat for preparing the manuscript.

I am indebted to my family for their help and encouragement without which it would have been much difficult for me to complete the present work.

Mohamed Ismail, K.

DECLARATION

I do hereby declare that this work entitled "**Calcium (Ca^{2+}) as the Signal Transducing Element in the Action of Insect Neuropeptide Hormones in *Iphita limbata* Stål (Pyrrhocoridae: Heteroptera)"** has been originally carried out by me under the guidance and supervision of Dr. M. Gokuldas, Professor and Head of the Department of Zoology, University of Calicut, and that this has not been submitted elsewhere for the award of any other degree or diploma.

C.U. Campus **Mohamed Ismail, K.**

15.04.2008

Dedicated to

My Parents

CONTENTS

Chapter 1

Introduction

INTRODUCTION

Insects constitute the largest group of organisms on the Earth and account for over 65% of all animal species. They have most successfully established themselves all over the globe in diverse habitats, wherever life is possible. Various factors have contributed to the successful existence and domination of the insect community. Among these factors, the efficiency in storage and utilization of food reserves is an important one. In this process, the role of hormones has been found to be similar to those found in higher vertebrates. As in vertebrates, the neuroendocrine system in insects is well developed and secretes several neuropeptides that are involved in the regulation of various pathways of intermediary metabolism. Most of these neuropeptides are synthesized in the neuronal complex formed of brain, corpora cardiaca (CC), corpora allata (CA) and suboesophageal ganglion. More than fifty neuropeptides have been identified from this complex of *Locusta migratoria* (Schoofs *et al*., 1997).

Adipokinetic hormone/red pigment concentrating hormone (AKH/RPCH) family is the most extensively studied group of insect neuropeptides. Adipokinetic hormones (AKH) are small peptides, synthesized and stored in the CC, from where they are released into the haemolymph for circulation. These peptides are primarily concerned with the mobilization and release of food reserves from the fat body.

Fat body is the principle storage organ of insects, comparable to the hepatopancreas in molluscs and crustaceans, and adipose and liver of vertebrates (Kilby, 1963; Price, 1973; Wyatt and Pan, 1978; Wyatt, 1980). Glycogen, proteins and lipids are the primary reserve food materials, of which lipids constitute a major portion. Triacylglycerol (TAG) forms the major storage lipid of insect fat body. During locomotion and other sustained activities, TAG reserves in the fat body are mobilized and released into the haemolymph mainly as diacylglycerol (DAG) and are transported to the sites of utilization. The mobilization, release, transport and utilization of lipids of fat body are regulated primarily through the mediation of the AKH. Other major functions of this pleiotropic hormone include the regulation of flight muscle metabolism, myostimulation, inhibition of synthesis of proteins, lipids and RNAs etc.

It has been shown that in most of the AKH-induced actions in insects, cyclic adenosine monophosphate (cAMP), calcium ion (Ca^{2+}) and inositol 1,4,5-trisphosphate (IP_3) are involved in the signal transduction, among which Ca^{2+} is found to be a major second messenger (Vroemen *et al*., 1998). The importance of both extracellular and intracellular Ca^{2+} were studied in various Ca^{2+}-mediated actions of AKH peptides. But the relative importance of extracellular and intracellular Ca^{2+} was not well established with regard to AKH-induced lipid mobilization and release from the fat body of insects. The previous works in this area were restricted to actively flying insects such as the

locusts (*Schistocerca gregaria* and *L. migratoria*) (Spencer and Candy, 1976; Wang *et al.*, 1990) and the moth (*Manduca sexta*) (Arrese *et al*., 1999). The data obtained from such studies were insufficient to draw a general picture on the modulating effect of Ca^{2+} on lipid release from the fat body of insects with different modes of locomotion like flight, swimming, crawling, walking and running.

In the present study, the phytophagous heteropteran bug, *Iphita limbata* was selected as a model insect to study the role of calcium (Ca^{2+}) on modulating the lipid mobilizing action of insect neuropeptides. Insects, in general, have been considered to be ideal models to study the metabolism of animals as their fat body, though simple in structure, is a metabolically complex tissue. Moreover, they share several aspects in common with both lower organisms and higher animals such as vertebrates including human beings. For example, the key enzyme necessary for lipid mobilization in insects, triacylglycerol lipase has several properties common with the vertebrate hormone-sensitive lipase which catalyzes the rate-limiting step in the mobilization of adipose tissue fatty acids. Therefore, any investigation on the modulation of lipid mobilisation in insect may facilitate our general understanding of the involvement and importance of second messengers in the signal transduction of hormones related to the mobilization of food reserves. In addition to that, a detailed knowledge of the signal cascade involving Ca^{2+} may be useful in the development of agonists and antagonists that could be

employed to derail key physiological processes causing deleterious effects and may be used to check the harmful insect population.

The major objectives of the present investigation were as follows.

I. Partial characterization of adipokinetic factors in the crude extract of brain-corpora cardiaca-corpora allata complex (brain-CC-CA complex) of *I. limbata* using reverse-phase high performance liquid chromatography (RP-HPLC) and subsequent bioassays.

II. To study the pharmacological effects of the following on the release of lipids from the fat body of *I. limbata*.

- Calcium (as $CaCl_2$)
- Synthetic *Locusta migratoria* adipokinetic hormone-I (Lom-AKH-I) in the presence/absence of calcium
- Crude extract of brain-CC-CA complex of *I. limbata* in the presence/ absence of calcium
- Calcium ionophore, A23187
- Calcium channel blockers, lanthanum (La^{3+}) and cadmium (Cd^{2+})
- Calcium chelator, EGTA and
- Internal calcium mobiliser, thimerosal.

Chapter 2

Review of Literature

REVIEW OF LITERATURE

INSECT HORMONES

Hormones are chemical messengers that in co-ordination with the nervous system regulate various vital activities of insects during each and every stage of their life cycle. The diversity, multiplicity and pleiotropic nature of insect hormones compensate the relatively less organized nervous system of insects to promote the interaction between cells and thereby to increase their information handling capacity. When compared to the rather simple organization of the body, insects are provided with a wide variety of hormones.

Insect hormones belong to three main groups namely lipophilic hormones, biogenic amines and peptide hormones.

Lipophilic Hormones

They are liposoluble, membrane permeant hormones which penetrate into cells and interact with the intracellular receptors which in turn penetrate the nucleus to evoke long term effects like specific mRNA synthesis (McEwan, 1991). This group includes moulting hormone and juvenile hormone.

Moulting hormone (MH) or ecdysone is a steroid hormone secreted by the prothoracic glands (PG) of insects (Smith, 1985; Sehnal, 1989). It was the first insect hormone to be structurally identified. Over 60 different analogs of the moulting hormone have been isolated from different insects (Klowden, 2002) and this group of steroid hormones is generally described as ecdysteroids.

Even though ecdysone is primarily concerned with moulting and metamorphosis of insects (Smith *et al*., 1985), it is also involved directly or indirectly in the regulation of lipid metabolism. Arnold and Regnier (1975) reported that ecdysteroids have some effects on lipid flux. Ecdysone-controlled synthesis of the female vitellogenin was reported in *Lucilia caesar*, *Calliphora erythrocephala* (De Loof, 1981) and in *Musca domestica* (Adams *et al*., 1985).

Juvenile hormones (JH), acyclic sesquiterpenoid epoxides (Raabe, 1989) secreted by CA, control reproduction and development in most of the insects (Aucoin *et al*., 1987). The existence of JH was demonstrated as early as 1936 in *Rhodnius prolixus* (Wigglesworth, 1936). But Pfeiffer (1945) was the first to demonstrate that CA contains a hormone which regulates lipid metabolism in insects. Following this report, the role of CA as a source of hormone regulating lipid metabolism was confirmed in the American cockroach, *Periplaneta americana* (Bodenstein, 1953), the migratory locust, *L. migratoria* (Strong, 1968) and in the desert locust, *S. gregaria* (Walker and Bailey, 1971). A decrease in haemolymph lipid titer was demonstrated after allatectomy in *P. americana* by Vroman *et al*. (1965), *S. gregaria* by Odhiambo (1966), *Spodoptera littoralis* by El-Ibrashy and Boctor (1970), *L. migratoria* by Lafon-Cazal and Morandini (1982) and in *Chrysocoris stolli* by Saha *et al*. (1986).

Biogenic amines

They are first messengers derived from amino acids like tyrosine and tryptophan. The important biogenic amines of insects are serotonin

(indolamine), epinephrine, dopamine (catecholamines) and octopamine (phenolamine) (Raabe, 1989). These amines transduce their signal through plasma membrane receptors and second messengers like cAMP and Ca^{2+} (Orchard *et al*., 1983 a, b; Downer *et al*., 1984; Pimley, 1985).

Serotonin-secreting aminergic neurons are abundant in optic lobes and suboesophageal and last abdominal ganglion. This biogenic amine is involved in stimulating the activity of Malpighian tubules (MT) in *Carausius morossus, R. prolixus* (Maddrell *et al*., 1969), *Aedes taeniorhynchus* (Maddrell and Phillips, 1978), *Calliphora vicina* (Schwartz and Reynolds, 1979) and *Papilio demodocus* (Nicolson and Millar, 1983) and in contractions of the oviduct in *Tabanus proximis* (Cook, 1981) and the cricket, *Gryllus bimaculatus* (Sefiani, 1986).

Epinephrine- and dopamine-secreting neurons are mainly located in the pars intercerebralis (Klemm and Flack, 1978). They are also present in protocerebrum, deutocerebrum and tritocerebrum (Mercer *et al*., 1983; Viellemaringe *et al*., 1984). Epinephrine is involved in fluid absorption by the midgut (Rafaeli *et al*., 1984) and dopamine is involved in fluid secretion by MT (Nicolson and Millar, 1983), lipid synthesis inhibition (Pimley, 1984) and oviduct contraction leading to oviposition (Sefiani, 1986).

Octopamine, the monohydroxyphenolic analogue of noradrenaline, is secreted by neurons, mainly in the ventral nerve cord (Evans and O'Shea, 1978; Davenport and Wright, 1986). They are also found in the calyces of the

mushroom bodies of cockroaches and bees (Mercer *et al*., 1983; Davenport and Wright, 1986). Octopamine regulates several activities of insects including hypertrehalosaemia in *P. americana* (Downer, 1979), hyperlipaemia in locusts (Orchard *et al*., 1982; Orchard and Lange, 1983 a), inhibition of lipid synthesis in *Glossina morsitans* (Pimley, 1984), myotropic contraction of the oviduct of *G. bimaculatus* (Sefiani, 1986), release of adipokinetic hormone from neurosecretory cells (NSC) of CC in *L. migratoria* (Pannabecker and Orchard, 1987) and transcription of genes (Amstrong and Robertson, 2006).

Peptide hormones

Include short peptides, polypeptides and long polypeptides (proteins) which are secreted by the brain, ventral nerve cord and CC. They transduce their signal through second messengers like cyclic guanosine monophosphate (cGMP), cAMP, IP_3, DAG and Ca^{2+}.

Proctolin, the first insect neuropeptide identified (Brown, 1975), isolated from the hindgut of the *P. americana* is a pentapeptide with the amino acid sequence Arg-Tyr-Leu-Pro-Thr (Starratt and Brown, 1975). It is one of the highly conserved peptide hormones of insects (Gäde, 2004). Proctolin-secreting neurons are located in the thoracic and abdominal ganglia of *P. americana* (Bishop and O'Shea, 1982) and in the whole central nervous system of *S. gregaria* (Keshishian and O'Shea, 1985). This peptide evokes or increases the contractions of the muscles of hindgut (Brown, 1975), foregut (Cook and Holman, 1978), body wall (Irving and Miller, 1980), oviduct (Cook, 1981;

Lange *et al*., 1986; Sefiani, 1986) and antennal heart (Hertel *et al*., 1985)**.** Proctolin also acts as a releasing factor for AKH and JH in locusts (Clark *et al*., 2006). Calcium ion is the major second messenger in mediating proctolin actions **(**Cook *et al*., 1975; Lange *et al*., 1987).

Diuretic peptides of insects increase water loss either by increasing MT secretion or by inhibiting fluid reabsorption from the hindgut (Gäde, 2004). Four classes of neuropeptides show diuretic activity. They are the following.

Arginine vasopressin like insect diuretic hormone (AVP-IDH) was the first diuretic peptide to be isolated and characterized in insects. It was extracted from suboesophageal and thoracic ganglia of *L. migratoria* and was shown to be an antiparallel dimer of the primary sequence Cys-Leu-Ile-Thr-Asn-Cys-Pro-Arg-Gly-amide (Proux *et al*., 1987).

Corticotropin-releasing factor-related diuretic hormones (CRF-related DH) have been isolated from locusts, crickets, cockroaches, termites, tenebrionid beetles, sphingid moths, culicid mosquitoes and houseflies (Coast *et al*., 2002). This neuropeptide family (CRF-related DH family) consists of about 15 members with amino acid residues ranging from 30 to 47 and brings in an increase of urine secretion by MT (Gäde, 2004). They are secreted chiefly by the NSC of pars intercerebralis of the brain (Mordue and Goldsworthy, 1969; Morgan and Mordue, 1983). The effects of CRF related DH of *Drosophila melanogaster* (Drome-DH_{44}) and *Tenebrio molitor* are mediated through cAMP (Cabrero *et al*., 2002; Wiehart *et al*., 2002).

Insect (myo)kinins is another class of diuretic peptides which have diuretic activity on MT. They are originally isolated from the extracts of whole heads of *Leucophaea maderae* (Holman *et al*., 1991). Different kinins have been fully sequenced from *Acheta domesticus, Culex salinarius, Helicoverpa zea*, *P. americana*, *M. domestica*, *D. melanogaster* and *L. migratoria* (Coast *et al*., 2002). Structurally, the kinin family is characterized by the C-terminal pentapeptide sequence of Phe-Xaa^1-Xaa^2-Trp-Gly-amide where Xaa^1 can be Asn, His, Phe, Ser or Ala and Xaa^2 can be Ser, Pro or Ala (Gäde, 2004). Generally kinins enhance the production of IP_3, which in turn releases Ca^{2+} from IP_3-sensitive stores (Cady and Hagedorn, 1999; Pollock *et al*., 2003).

Cardioacceleratory peptides **(CAP)** is yet another group of diuretic peptides of insects. The first CAP to be identified was isolated from *M. sexta* (Manse-CAP_{2b}) and has the amino acid sequence pGlu-Leu-Tyr-Ala-Phe-Pro-Arg-Val-amide (Gäde, 2004). It acts on MT. Similar CAP were identified and sequenced from *D. melanogaster* and other insects but are often called periviscerokinins (Wegener *et al*., 2002; Kean *et al*., 2002). Similar to kinins, CAP use both IP_3 and Ca^{2+} for signal transduction (Coast *et al*., 2002; Pollock *et al*., 2003).

Antidiuretic peptides (ADP) of insects are of two types: those that inhibit MT secretion and those that stimulate fluid reabsorption by the ileum or rectum (Gäde, 2004). Antidiuretic peptides acting on MT are present in the Colorado potato beetle, *Leptinotarsa decemlineata* (Lavigne *et al*., 2001), the

forest ant, *Formica polyetena* (Laenen *et al*., 2001) and in the yellow mealworm, *T. molitor* (Eigenheer *et al*., 2002, 2003). The ADP isolated from *T. molitor* are code named as Tenmo-ADFa and -ADFb and are structurally non-blocked peptides. Both peptides use cGMP as the second messenger (Eigenheer *et al*., 2002, 2003).

Neuroparsins isolated from the NSC of CC of *L. migratoria* are ADP acting on the hindgut. The longest one contains 83 amino acids and smallest one 78. They make use of IP_3 and Ca^{2+} for signal transduction (Gäde *et al*., 1997).

Ion-transport peptide (ITP) is another ADP which acts on the hindgut. Homologues of ITP are found in *S. gregaria* (Phillips and Audsley, 1995), *Bombyx mori, L. migratoria* and *D. melanogaster* (Phillips *et al*., 2001). It is secreted by the intrinsic cells of CC (Coast *et al*., 2002). The ITP isolated from *S. gregaria* (Schgr-ITP) has 72 amino acid residues (Meredith *et al*., 1996) and shares about 40% sequence identity with the crustacean hyperglycemic hormone (Lacombe *et al*., 1999). The ITP from *B. mori* and *D. melanogaster* are quite different from Schgr-ITP, whereas that from *L. migratoria* and *Pachnoda sinuata* are identical to Schgr-ITP and others are suggested to have only minor changes in the sequence (Phillips *et al*., 2001). Cyclic AMP is involved in the action of Schgr-ITP (Gäde, 2004).

Chloride transport-stimulating hormone (CTSH) isolated from the CC of desert locust is another ADP acting on the hindgut of insects. Its mode of

action on ion transport mechanism has been found to be via cAMP (Phillips and Audsley, 1995).

Pheromone biosynthesis activating neuropeptide (PBAN) activates pheromone glands for biosynthesis of sex pheromones (Raina and Klun, 1984). It was first isolated from the suboesophageal ganglion of the moth, *H. zea* (Raina *et al*., 1989). Later, two PBAN were isolated from *B. mori* (Bom-PBAN-I and -II) (Kitamura *et al*., 1989, 1990) and one from the gypsy moth, *Lymantria dispar* (Lyd-PBAN) (Masler *et al*., 1994). PBAN is a 33-40 amino acid peptide that has a core sequence of Phe-Ser-Pro-Arg-Leu-NH_2 at C-terminus which conveys pheromonotropic activity (Ozawa and Matsumoto, 1996). Calcium acts as the signal transducing element of PBAN action (Ozawa *et al*., 1995).

Prothoracicotropic hormone (PTTH) or **brain hormone** was first isolated and identified from adult male *B. mori* (Nagasawa *et al*., 1984). It is produced by the NSC of the brain (Gibbs and Riddiford, 1977; Furtado, 1979) and is found to stimulate PG to synthesize ecdysone (Bollenbacher and Granger, 1985). In the tobacco horn worm, *M. sexta* and perhaps in all lepidoptera, PTTH is released from CA (Agui *et al*., 1980), but in majority of other insects, the major release site of PTTH is corpora cardiaca (CC) (Raabe, 1989). PTTH is known to exist in two different molecular sizes: big PTTH and small PTTH. Both hormones stimulate ecdysteroid secretion by PG, but at different stages of development (Bollenbacher *et al*., 1984; O'Brien *et al*., 1986). In *M. sexta*, big PTTH is with a molecular weight of ~25.2 kDa and small PTTH is with ~7 kDa

(Bollenbacher *et al*., 1984; Muehleisen *et al*., 1993) whereas in *B. mori*, big one is 30 kDa and small one is 4 kDa (Raabe, 1989). The PTTH are species specific, for example, the hormones isolated from *M. sexta* and *B. mori* are dissimilar and the PTTH of *B. mori* has no activity in *M. sexta* or in *D. melanogaster* (Gilbert *et al*., 1996). Cyclic AMP and Ca^{2+} are involved in the signal transduction of both big and small PTTH (Smith *et al*., 1985, 1986; Meller *et al*., 1988; Hayes *et al*., 1995; Gu *et al*., 1998).

Allatotropins are synthesised in the NSC of brain (Gadot and Applebaum, 1985; Gadot *et al*., 1987; Kataoka *et al*., 1989). They stimulate CA to synthesize and release JH (Gadot *et al*., 1987; Gilbert *et al*., 2000; Stay, 2000). They have been isolated from the tobacco hornworm, *M. sexta*, (Kataoka *et al*., 1989), the tomato moth, *Lacanobia oleracea* (Audsley *et al*., 1998) and the fall armyworm, *Spodoptera frugiperda* (Oeh *et al*., 2000). The allatotropin of *M. sexta* (Mas-AT) has the amino acid sequence Gly-Phe-Lys-Asn-Val-Glu-Met-Thr-Ala-Lys-Gly-Phe (Kataoka *et al*., 1989).

Allatostatins are myotropins which inhibit JH biosynthesis by CA (Kikukawa *et al*., 1987). They also act as myoinhibiting peptides (Schoofs *et al*., 1997). These neuropeptides were isolated from the brain and CC-CA complex (Rankin *et al*., 1986; Veelaert *et al*., 1995). Allatostatins constitute the largest family of known arthropod neuropeptides and more than 150 different arthropod allatostatins have been identified and they have been characterized by the C-terminal signature Tyr/Phe-Xaa-Phe-Gly-Leu/Ile-amide (Mousley *et al*., 2005).

In insects, allatostatins have been identified from *S. gregaria*, *P. americana*, *Diploptera punctata*, *Blatella germanica*, *Calliphora vomitoria*, *M. sexta* and *G. bimaculatus* (Belles *et al*., 1994; Stay *et al*., 1994; Lorenz *et al*., 1995; Veelaert *et al*., 1996). Cyclic AMP is a part of signal transduction of allatostatin from *D. punctata* (Meller *et al*., 1985; Aucoin *et al*., 1987).

Eclosion hormone (EH) is secreted by the ventrally located NSC of the brain in response to falling ecdysteroid titer (Truman, 1985; Kingan and Adams, 2000), and is released into the haemolymph through CC-CA complex (Copenhaver and Truman, 1986). The EH control eclosion behaviour (Truman and Riddiford, 1970; Truman, 1971) and the muscle degeneration that follows eclosion (Truman, 1973). Four closely related eclosion hormones, EH-I, -II, -III and -IV were purified from *B. mori* (Kono *et al*., 1991) and one EH from *M. sexta* (Terzi *et al*., 1998). In *M. sexta* it is a polypeptide with 62 amino acid residues (Kataoka *et al*., 1987). Cyclic GMP have been reported to be the second messenger involved in the hormonal action of EH (Truman *et al*., 1979; Schwartz and Truman, 1984; Shibanaka *et al*., 1991, 1994; Morton and Simpson, 2002).

Bursicon is a neurohormone secreted by the NSC of central nervous system especially the thoracic and abdominal ganglia of the ventral nerve cord (Taghert and Truman, 1982; Honegger *et al*., 2002) and is produced by the same neurons that produce crustacean cardioactive peptide (CCAP) (Honegger *et al*., 2004). This hormone coordinates events such as cell death of wing epidermis

and wing expansion and the subsequent tanning and hardening of cuticle after ecdysis (Kimura *et al*., 2004; Dewey *et al*., 2004). The bursicons identified from *T. molitor*, *C. erythrocephala, P. americana, G. bimaculatus* and *L. migratoria* have a molecular mass of 30 kDa (Kaltenhauser *et al.*, 1995; Kostron *et al*., 1995). This neurohormone is a heterodimer and is the first heterodimeric cystine knot hormone to be detected in insects and consists of two proteins encoded by the genes *burs* and *pburs* (partner of *burs*) (Luo *et al*., 2005). Bursicon of diverse insects initiates tanning in neck ligated flies (Cottrell, 1962; Fraenkel and Hsiao, 1962) suggesting the conservation of at least some sequences of bursicon in different insects. Honegger *et al*. (2002) suggested that the 12 amino acid sequence is conserved even among species that are distantly related. The activation of cAMP/PKA signalling pathway is required for the transduction of the hormonal signals of bursicon (Kimura *et al*., 2004).

Cardioacceleratory hormones (CAH) are a class of myotropic neuropeptides which can accelerate insect heart beat. It includes neurohormone D (Gersch *et al*., 1960), CCAP (Stangier *et al*., 1989) and corazonin (Veenstra, 1989).

Neurohormone D (NHD) or *Periplaneta* CAH-I was the first CAH to be separated from CC of *P. americana* (Gersch *et al*., 1960). It has the primary structure, pGln-Val-Asn-Phe-Ser-Pro-Asn(Gly/Trp) (Baumann and Penzlin, 1984). NHD is a hypertrehalosaemic hormone and is now named as *Periplaneta*-HTH-I (Goldsworthy *et al*., 1997). Later a second CAH

(*Periplaneta* CAH-II) was isolated from the CC of the same insect and has the structure pGln-Leu-Thr-Phe-Thr-Pro-Asn-Thr-NH_2 (Siegert and Mordue, 1986). Just like NHD, *Periplaneta*-CAH-II also has hypertrehalosaemic effect and is named as *Periplaneta*-HrTH-II.

Crustacean cardioactive peptide (CCAP) was first isolated and identified from the shore crab, *Carcinus maenas* (Stangier *et al*., 1987). Since then CCAP was isolated from other insects such as *L. migratoria* (Stangier *et al*., 1989), *M. sexta* (Cheung *et al*., 1992), *T. molitor*, *Spodoptera eridania* (Furuya *et al*., 1993) and *S. gregaria* (Veelaert *et al*., 1997). It accelerates heart beat in *M. sexta* (Schoofs *et al*., 1997) and induces the release of AKH from the brain complex in *S. gregaria* (Veelaert *et al*., 1997) and the contraction of oviduct in *L. migratoria* (Donini *et al*., 2001). The identification of CCAP as a releasing hormone for AKH provides the first evidence for the existence of neuropeptides in invertebrates that act as hormonal releasing factors for other peptide hormones (Schoofs *et al*., 1997). The CCAP isolated from *L. migratoria*, *M. sexta*, *S. gregaria*, *T. molitor* and *S. eridania* has the amino acid sequence identical to the CCAP isolated from *C. maenas* (Stangier *et al*., 1989; Cheung *et al*., 1992; Furuya *et al*., 1993; Veelaert *et al*., 1997). Schoofs *et al*. (1997) suggested that the primary structure of CCAP is conserved in different insect species. Calcium is the second messenger of CCAP-induced oviduct contraction in *L. migratoria* (Donini and Lange, 2002).

Corazonin is another cardioactive peptide that stimulates the heart rhythm. It was first isolated from the CC of *P. americana* and has the primary structure of pGlu-Thr-Phe-Glu-Tyr-Ser-Arg-Gly-Trp-Thr-Asn-NH_2 (Veenstra, 1989). Later this peptide was isolated from other insects including *Nauphoeta cinerea*, *Apis mellifera*, *S. gregaria*, *M. sexta*, *C. morosus* and from members of Mantophasmatodea (Veenstra, 1991; Verleyen *et al*., 2006; Predel, *et al*., 2007). Only a single isoform of this peptide occurs in any particular species of insects studied so far. Corazonin, however, has not been reported from any Coleopteran species (Predel *et al*., 2007). Very few modifications of [Arg^7]-corazonin originally isolated from cockroaches, are known, for example, [His^7]-corazonin which is found in certain locusts, stick insects and wasps, [Thr^4, His^7]-corazonin from *A. mellifera*, [Tyr^3, Glu^7, Glu^{10}]-corazonin from bumble bee and [His^4, Glu^7]-corazonin from South African members of Mantophasmatodea (Verleyen *et al*., 2006; Predel *et al*., 2007). Corazonin is secreted by the pars lateralis of the protocerebrum and stored in the storage lobes of CC (Predel *et al*., 2007).

Diapause hormone (DPH) was first reported in *B. mori* by Hasegawa (1951). It induces embryonic diapause in the eggs of *B. mori* (Yamashita and Hasegawa, 1966; Yamashita, 1996). The DPH isolated from *B. mori* has 24 amino acid moieties (Imai *et al*., 1991). The DPH is secreted by the NSC of suboesophageal ganglion, brain and CC-CA complex of *B. mori* (Yamashita and Hasegawa, 1966; Takeda and Ogura, 1976; Hasegawa and Shimuzu, 1990; Yamashita, 1996). The DPH is also found in the suboesophageal ganglion of

other diapausing species such as *L. dispar, Antheraea yamala* (Fukuda, 1951; Hasegawa, 1951) and in species without egg diapause but with pupal diapause, e.g., *Phalaenoides glycinae* (Andrewartha *et al*., 1974). It is also present in the brain, CC and CA of the common armyworm, *Leucania separata* (Ogura and Saito, 1973), *P. americana* (Takeda, 1977) and *L. migratoria* (Takeda and Girardie, 1985).

Hyperglycemic/hypertrehalosaemic hormone (HGH/HTH) was the first peptide hormone reported from the CC of *P. americana* (Steele, 1961). Two peptides with hypertrehalosaemic activity have been purified from the CC of this insect (Scarborough *et al*., 1984). Similar peptides have been isolated from various insects including *Blaberus discoidalis* (Hayes *et al*., 1986), *C. morosus* (Gäde and Rinehart, 1987 a; Gäde *et al*., 1992), *H. zea* (Jaffe *et al*., 1988) and *Phormia terraenovae* (Gäde *et al*., 1990). This hormone can be compared to the glucagon of vertebrates. Hypertrehalosaemic hormones are either blocked octapeptides or decapeptides (Gäde, 1992 b) which cause activation of fat body phosphorylase (Steele, 1963; Wiens and Gilbert, 1967) through the mediation of Ca^{2+} (Orr *et al*., 1985; Park and Keeley, 1996).

Hypoglycemic/Hypotrehalosaemic hormone (HoGH/HoTH) was detected in the NSC of brain in insects such as the honey bee (Dixit and Patel, 1964) and the blowfly (Norman, 1975). Tager *et al*. (1976) identified insulin-like material in the extract of the CC-CA complex from *M. sexta*. The insect insulin molecule is similar to mammalian insulin. The HoTH isolated from

Tabanus has the primary sequence of pGlu-Leu-Thr-Phe-Thr-Pro-Gly-Trp-Gly-Tyr-NH_2 (Kramer *et al*., 1982).

ADIPOKINETIC HORMONE

In 1969, Mayer and Candy showed that the peptide contained in CC of *S. gregaria* controlled mobilization and release of lipids from the fat body. They named this peptide as adipokinetic hormone. Simultaneously, Beenakkers (1969) reported a similar factor from the CC of *L. migratoria*, which caused a similar lipid release. The primary structure of this AKH (AKH-I) was elucidated by Stone *et al*. (1976). The locust AKH was found to have cross reactivity in the shrimp, *Pandalus borealis* where it elicited the activity of red pigment concentrating hormone (RPCH) (Fernlund, 1974) and the molecular structure of AKH closely resembles that of the RPCH of prawns (Fernlund and Josefsson, 1972; Fernlund, 1974; Stone *et al*., 1976; Mordue and Stone, 1976). These two sets of hormones were thus included in the AKH/RPCH family (Orchard, 1987).

All AKH are hydrophobic compounds and are stable at high temperature. Adipokinetic hormones are detected and isolated from almost all major orders of insects and is one of the most extensively investigated insect neurohormone of recent years (Auerswald *et al*., 2005; Gäde *et al*., 2005, 2006, 2007 a, b). Most insects have only one AKH peptide, but some species have two, three or four isoforms (Stone *et al*., 1976; Siegert *et al*., 1985, 2000; Oudejans *et al*., 1991; Gäde *et al*., 1994 a; Siegert, 1999).

Source of AKH

The AKH are synthesised in the intrinsic NSC of CC. In locusts, but not in other insects, these intrinsic cells form a separate lobe called the glandular lobe and most of the AKH is located in this lobe (Goldsworthy *et al*., 1972 a, b; Hekimi and O'Shea, 1985). The adipokinetic cells are neuron like unipolar cells with short cell processes (Cassier and Fain-Maurel, 1970; Orchard and Shivers, 1986). The glandular lobes of CC contain approximately 6,000 to 10,000 adipokinetic cells (Hekimi and O'Shea, 1987; Schulz-Aellen *et al*., 1989; Hekimi *et al*., 1989). These cells contain large number of electron dense granules of 200 to 600 nm diameters (Rademakers and Beenakkers, 1977; Krogh and Norman, 1977) and AKH reside in these dense granules (Stone and Mordue, 1979). Despite the fact that in the glandular part of CC of *L. migratoria* three AKH have been identified, morphologically only one type of adipokinetic cells has been demonstrated to be present in this part of CC (Diederen *et al*., 1987).

The presence of AKH-I in the locust brain is indicated by immunocytochemistry (Schooneveld *et al*., 1983) and has been substantiated by Moshitzky *et al*., (1987 a, b) and Bray *et al*., (1993). However, the quantity present in the brain accounts to only 10% of that found in the CC. Similarly, using radioimmunoassay, *Manduca* brain was found to contain *Manduca*-AKH (Fox and Reynolds, 1990). AKH-like material is found in other insect neural tissues (Schooneveld *et al*., 1983; Ziegler *et al*., 1988) but only in small

quantities. The fourth AKH identified from *L. migratoria* was found in the storage lobe of CC and hence is presumed that they are probably synthesized in the brain (Siegert, 1999).

Synthesis and release of AKH

Corpora cardiaca is the site of synthesis, storage and release of AKH **(**Stone *et al*., 1976; Siegert *et al*., 1985; Hekimi and O'Shea, 1987, 1989; Oudejans *et al*., 1991; Schoofs *et al*., 1993). The molecular aspects of biosynthesis of AKH were studied in a few insects, especially in locusts (Hekimi and O'Shea, 1987; Rayne and O'Shea, 1994; Bogerd *et al*., 1995). The NSC of CC in *S. gregaria* express two co-localised transcripts which are translated into two preprohormones required for AKH-I and -II biosynthesis (Fischer-Lougheed *et al*., 1993). In *L. migratoria* the signals for the mRNA of all the three AKH preprohormones are co-localised in the NSC of CC (Bogerd *et al*., 1995). The AKH preprohormones have a general organization consisting of a signal peptide, one single copy of AKH, amidation and processing sites followed by another peptide, the AKH precursor related peptide (APRP) (Bogerd *et al*., 1995; Gäde, 2004). The enzymatic removal of the signal sequences from the preprohormones generates pro-AKH-I, -II and -III (Rayne and O'Shea, 1994). The AKH-I and -II prohormones are structurally very similar whereas that of AKH-III is remarkably different (Bogerd *et al*., 1995). Prior to the further processing, the precursors of AKH-I and -II form dimers at random through the formation of cysteine bridge resulting in a mixture of one

heterodimeric (AKH-I/II) and two homodimeric (AKH-I/I, AKH-II/II) prohormones. This dimerisation is rather a unique phenomenon which was first established for AKH of *S. gregaria* (Hekimi and O'Shea, 1989; Hekimi *et al*., 1989). Within the dimeric constructs, the AKH sequences are separated from the 28 residue APRP sequence by classical processing sites (Baggerman *et al*., 2003) followed by amidation which results in the generation of bioactive hormones as well as three (two homodimeric and one heterodimeric) APRP (Van der Horst *et al*., 2001; Diederen *et al*., 2002; Oudejans and Van der Horst, 2003). Available data suggest that the processing of the prehormones of AKH-I and -II in *L. migratoria* is similar to that in *S. gregaria* (Diederen *et al*., 2002). The biosynthesis of AKH-III from its prohormone was discovered by Huybrechts *et al*. (2002). AKH-III prohormone also undergoes dimerisation (homodimer) and subsequent proteolytic cleavage that result in AKH-III and a fourth APRP.

The AKH prohormones are synthesized in the rough endoplasmic reticulum (ER) of the NSC bodies, transported to the Golgi complex and packaged into secretory granules at the trans-Golgi network (Diederen *et al*., 2002). Proteolytic processing of the prohormones to bioactive AKH is considered to take place in a post-trans-Golgi compartment, presumably the secretory granules (O'Shea and Rayne, 1992; Van der Horst *et al*., 2001; Diederen *et al*., 2002; Oudejans and Van der Horst, 2003). The secretory granules which have a mean diameter of about 300 nm are transported into the

cell processes, where they are either stored or released by exocytosis into the haemolymph (Diederen *et al*., 1987, 1992, 1993; Jansen *et al*., 1989). In *L. migratoria,* the synthesis of prohormones of the AKH, their packaging into secretory granules and their processing to the bioactive hormones are completed in less than 75 min (Oudejans *et al*., 1990, 1991).

Flight is the primary stimulus and probably the only natural stimulus for the release of AKH in locusts (Mayer and Candy, 1969; Cheeseman and Goldsworthy, 1979; Orchard and Lange, 1983 a, b; Diederen *et al*., 2002). Release of AKH upon flight was established by using biological assays (Cheeseman and Goldsworthy, 1979), but the precise stimuli responsible for initiating release of AKH during flight are unknown (Goldsworthy, 1983). Receptors associated with wing movements or wind receptors, or change in metabolite levels, could be involved but none has been established (Goldsworthy, 1976, 1983). Both neuronal and hormonal factors are involved in the release of AKH from CC.

In locusts, the release of AKH is under the synaptic control of axons within the nervi corporis cardiaci (NCC)-I and -II and stimulation of NCC-II induces the release of AKH from CC (Orchard and Loughton, 1981). Stimulation of NCC-I alone does not cause release, but it potentiates NCC-II stimulated AKH release. Thus the axons of NCC-I play no part in the initiation of hormone release, but could play a modulatory role (Rademakers, 1977; Orchard and Loughton, 1981). Pannabecker and Orchard (1987) reported that

aminergic neurons pass via the nerve tract, NCC-II from the protocerebrum and innervate the glandular lobe. These neurons make synaptic contact with the NSC and regulate the release of AKH.

According to Orchard *et al*. (1993), octopamine is involved in AKH release as the natural neurotransmitter of the secretomotor neurons that make synaptic contact with the adipokinetic cells. Diederen *et al*. (2002) also suggested a humoral role for octopamine since the haemolymph titer of octopamine is known to increase three-fold at the onset of flight (Goosey and Candy, 1980; Goldsworthy, 1983). Passier *et al*. (1995) suggested that octopamine only potentiates the release initiating factors that stimulates adipokinetic cells resulting in AKH release and that it cannot induce the AKH release on its own. Dopamine, serotonin and tyramine also potentiate the AKH release in a similar fashion. Pannabecker and Orchard (1987) reported that an influx of extracellular Ca^{2+} (Ca^{2+}_{o}) into the NSC provides an essential trigger for initiating AKH release. Peptidergic releasing factors for AKH in *L. migratoria* are identified *in vitro* as tachykinins-I and -II (Lom-TK-I and-II) (Nässel *et al*., 1995) and CCAP (Veelaert *et al*., 1997; Flanigan and Gäde, 1999).

Adipokinetic hormone release can be artificially induced by certain insecticides (Samaranayaka, 1974; Singh and Orchard, 1982), anticholinesterases (Samaranayaka, 1976) and by proctolin administration (Clark *et al*., 2006).

Release of AKH *in vitro* has been found to get inhibited by several factors. For example, trehalose was shown to inhibit AKH release (Passier *et al*., 1997). The tetrapeptide FMRF-amide, the decapeptide Schisto FLRF amide (Vullings *et al*., 1998) and the locust myoinhibiting peptide, Lom-MIP (Harthoon *et al*., 2001) are also inhibitors of AKH release.

Structure of AKH

The first insect AKH to be isolated and characterised was the decapeptide AKH-I from the CC of the locusts, *L. migratoria* and *S. gregaria*. It has the amino acid sequence pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH_2 (Stone *et al*., 1976). AKH-I of *L. migratoria* (AKH-I-L) and *S. gregaria* (AKH-I-S) showed same retention time when chromatographed indicating again that their primary structures are identical (Siegert *et al*., 1985). This peptide has been denoted as Lom-AKH-I (*Locusta migratoria* adipokinetic hormone-I). A second AKH was isolated from the CC of both *S. gregaria* (Carlsen *et al*., 1979) and *L. migratoria* (Gäde, 1984) and are designated as AKH-II-S and AKH-II-L respectively. They are octapeptides and their structures are different in the two locust species (Siegert *et al*., 1985; Gäde *et al*., 1986).

AKH-II-S: pGlu-Leu-Asn-Phe-Ser-Thr-Gly-Trp-NH_2

AKH-II-L: pGlu-Leu-Asn-Phe-Ser-Ala-Gly-Trp-NH_2

A third AKH of *L. migratoria* (Lom-AKH-III) was isolated from the glandular lobes of the CC and sequenced by Oudejans *et al*. (1991). It has an

amino acid sequence: pGlu-Leu-Asn-Phe-Thr-Pro-Trp-Trp-NH_2. Recently a fourth AKH has been isolated and sequenced from the storage lobe of the CC of *L. migratoria* (Siegert, 1999). It is named as '*Locusta migratoria* hypertrehalosaemic hormone' (Lom HrTH) and has the primary sequence:

pGlu-Val-Thr-Phe-Ser-Arg-Asp-Trp-Ser-Pro-NH_2

Meanwhile, a large number of peptides were identified from the CC extracts of other insects. About 40 isoforms of this peptide hormone have been identified from different insect orders so far. Table II-1 shows the primary structures of AKH/RPCH family peptides isolated from the CC of various insects.

The AKH are small peptides with 8 to 10 amino acid residues (Ziegler *et al*., 1995; Gäde and Auerswald, 2003). All these peptides are with blocked termini; N-terminally blocked by a pyroglutamate (pGlu) residue and C-terminally amidated (Van der Horst, 2003). Köllisch *et al*. (2000) isolated and sequenced an unusual AKH from the butterfly *Vanessa cardui* (Vac-AKH-I). It has 11 amino acids with a non amidated C-terminus.

When amino acid analysis and sequencing were carried out, it was found that the different peptides of AKH/RPCH family show high degree of sequence homology. Almost all these peptides possess $pGlu^1$, Phe^4 and Trp^8 (Orchard, 1987), but in a few cases, phenylalanine at position 4 is replaced by tyrosine (Goldsworthy *et al*., 1997). In 11 of them first four amino acids from the

N-terminus are identical in having $pGlu^1$, Val^2, Asn^3 and Phe^4; in another 11 peptides, the first four are pGlu, Leu, Asn and Phe and nine of them have $pGlu^1$, Leu^2, Thr^3 and Phe^4 (See Table II-1). Conservative changes are observed; at position 2, Leu to Val, Ile, Lys, Phe and Tyr; at position 3, Asn to Thr and at position 5, Thr to Ser replacements are found. Most variability is seen in position 7 where 6 different amino acids are found. Substitution at position 6 can be proline, serine, alanine or threonine residue (Goldsworthy *et al*., 1997). In nona- and decapeptides, residue at position 9 is always glycine except in *L. migratoria* HrTH, where glycine is replaced by serine (See Table II-1).

Studies on the secondary structure of AKH showed the existence of a β-bend between residues 5 and 8 (Stone *et al*., 1978). Stability would be conferred on AKH by hydrogen bonding between residues 3 and 10 and between 5 and 8 (Mordue and Morgan, 1985) (Figure II-1).

Structure-activity relationship

Generally, binding of neuropeptide molecules to specific membrane bound receptor proteins in target cells is a prerequisite for exerting hormonal activity (Gomperts *et al*., 2003). The shape and chemical nature of the ligand plays an important role in receptor recognition (Nachman *et al*., 1993). The structures of the peptides of AKH/RPCH family have been found to be remarkably stable with only little changes by a few selective amino acids because of the interdependency of the hormone and receptor (Goldsworthy *et al*., 1997). Gäde (1990 a, b) proposed that different binding requirements exist

for the AKH/RPCH peptides with respect to their receptors in shrimps, locusts and cockroaches. In cockroach HTH, the blocked termini are important and the amino acids Phe, Pro and Trp at position 4, 5 and 8 are essential for receptor binding. Using natural and synthetic analogues of the peptides, Hayes *et al*. (1986), Ford *et al*. (1988) and Hayes and Keeley (1990) reported that $pGlu^{1}$, Phe^{4} and Trp^{8} are essential for the initial recognition of the receptor and maximal response. They also proposed that the N-terminal octapeptide region is more important in recognizing the receptor than the C-terminal dipeptide. But Minnifield and Hayes (1992) reported that $pGlu^{1}$ is not essential for the AKH for binding to the receptor. The $pGlu^{1}$-removed AKH from *Tabanus atratus* was successful in binding to the receptors of *Musca autumnalis*. In AKH-I of *L. migratoria*, a hydrophobic cluster of Leu^{2}, Phe^{4} and Trp^{8} is important for receptor interaction (Goldsworthy *et al*., 1990). The fact that, Phe at position 4 is more important for receptor interaction to act as adipokinetic, in locusts and *M. sexta* and hypertrehalosaemic in *P. americana* and *B. discoidalis* suggested that Phe is conserved in all AKH/RPCH peptides (Gäde, 1990 b; Hayes and Keeley, 1990; Fox and Reynolds, 1991). However, in *Melolontha* CC and *Onitis* CC-II (See Table II-1), it was found to be replaced by Tyr (Gäde, 1991 b, 1997).

Extensive studies have been made to analyze the relationship between structures and activities of AKH in relation to its lipid mobilizing (Stone *et al*., 1978; Mordue, 1980) and acetate uptaking actions (Lee and Goldsworthy,

1995). The results indicated that there should be atleast eight amino acid residues for any biological activity. All the active peptides were uncharged, though it is not clear, whether charge is significant or not. The $pGlu^1$ is conserved in all natural AKH and is absolutely essential for its biological activity. The removal of $pGlu^1$ in the AKH-I (Goldsworthy *et al*., 1997) and the replacement of $pGlu^1$ by hydroxyphenyl propionate in *B. discoidalis* HrTH (Bld-HrTH) made the peptides inactive (Hayes *et al*., 1994). But $pGlu^1$ in AKH-I can be replaced by hydroxyphenyl propionate without serious loss of potency in *L. migratoria*. Here, substitution of $pGlu^1$ of AKH-I by Pro or Ala has been found to severely reduce the potency, particularly for Ala^1-AKH in the lipid mobilization assays (Goldsworthy *et al*., 1997). An analogue of *M. sexta*-AKH in which pGlu has been replaced by Gly was completely inactive in *Manduca* larvae (Ziegler *et al*., 1991).

The chain length of the peptide is also significant in modifying the potency of AKH action. Increasing the peptide length by inserting one or more amino acids or decreasing the length by removal resulted in considerable decrease (100-3000 fold) in the potency of AKH-I and III (Goldsworthy *et al*., 1994, 1997; Lee *et al*., 1997).

In *M. sexta,* in addition to the standard AKH framework of $pGlu^1$, Phe^4, Trp^8 and the C-terminal amide, the residues Leu^2, Thr^3, Ser^6 and Gly^9 seem to be important for full adipokinetic activity (Fox and Reynolds, 1991). The side chain of Thr^5 is more critical than that of Thr^{10} for the biological activities of

AKH-I (Poulos *et al*., 1994). Velentza *et al*. (2000) reported that for the biological activity of AKH-I in locust, the position 4 requires phenyl ring in the side chain and position 8, an indole ring.

Periplaneta CAH-I has a sequence of pGlu-Val-Asn-Phe-Ser-Pro-Asn-Trp-NH_2 (Witten *et al*., 1984). Here the proline residue at position 6 is not essential for maximal activity. Similarly analogues that have single replacements at position 2 (Leu instead of Val) and 5 (Thr instead of Ser) are as active as the endogenous peptide. But amino acid residues at position 4 (Phe) and 7 (Asn) are essential for hormonal action (Gäde, 1992 a). In *S. gregaria*, unmodified AKH-II can stimulate both IP_3 and cAMP signalling pathways but substitution of phenylalanine at position 4 and/or tryptophan at position 8 by alanine produced peptides that were unable to stimulate either signalling pathways (Stagg and Candy, 1998).

Physiological roles of AKH

Adipokinetic hormone is a pleiotropic hormone performing multiple roles in insects' life. The actions of the adipokinetic family of peptides are quite diverse and certainly broader than what their name implies. The primary and classical function of hormones belonging to AKH/RPCH family is mobilization of food reserves from the fat body to provide energy substrates for various energy demanding processes. AKH induce hyperlipaemia (Mayer and Candy, 1969; Beenakkers, 1969; Goldsworthy and Mordue, 1989; Goldsworthy *et al*., 1997), hypertrehalosaemia (Gäde, 1981; Van Marrewijk *et al*., 1986; Siegert *et*

al., 1986; Oudejans *et al*., 1991) and hyperprolinaemia (Auerswald and Gäde, 1999 a) in various insects. In *L. migratoria* each of the 3 AKH (AKH-I, -II and -III) is capable of mobilizing both glycogen and TAG (Oudejans *et al*., 1992). In *M. sexta*, a single AKH regulates the mobilization of carbohydrates in larvae and lipids in adults (Ziegler, 1984; Ziegler *et al*., 1990) and in *P. sinuata*, one and the same endogenous AKH peptide, Mem-CC, have both hyperprolinaemic and hypertrehalosaemic activity (Auerswald and Gäde, 1999 a, b, 2000).

In insects which utilize carbohydrate as the source of energy, AKH activates glycogen phosphorylase (Gäde, 1981; McClure and Steele, 1981; Van Marrewijk *et al*., 1983, 1993) which in turn hydrolyses glycogen which is a prerequisite for the synthesis of trehalose, the insect haemolymph sugar (Van Marrewijk *et al*., 1993). In those insects which utilize either lipids or proline as the fuel, AKH activates TAG lipase (Tietz and Weintraub, 1978; Wang *et al*., 1990; Arrese *et al*., 1996; Van der Horst, 2003; Gäde and Auerswald, 2003) resulting in the hydrolysis of TAG into DAG and fatty acids. In lipid utilizing insects, DAG are released into the haemolymph which are taken into the tissue where they are metabolized, while in proline utilisers, fatty acids formed due to AKH action undergo β-oxidation to generate acetyl CoA, a precursor for proline synthesis (Auerswald and Gäde, 2001 a).

In addition to the mobilization of lipids, the adipokinetic effects of AKH include lipid transport, unloading of lipids into the tissues and lipid synthesis inhibition (Pimley and Langley, 1981; Goldsworthy, 1983; Goldsworthy and

Wheeler, 1984; Wheeler and Goldsworthy, 1985 a; Gokuldas *et al*., 1988; Gokuldas, 1989). After mobilization, DAG are transported as part of a high molecular weight lipoprotein termed lipoprotein A^+. AKH helps in the transformation of a high density lipoprotein (HDLP) termed A_{yellow} or lipophorin to a low density lipoprotein (LDLP) A^+ or activated lipophorin by promoting the reversible association of a low molecular weight protein C_L with A_{yellow} lipoprotein and DAG (Orchard, 1987; Van Heusden and Law, 1989).

During lipid transfer to the tissues a lipophorin lipase hydrolyses the DAG carried by A^+ lipoprotein, releasing free fatty acids which are taken up by the tissues (Wheeler *et al*., 1984; Wheeler and Goldsworthy, 1985 b). Lipophorin lipase appears to be regulated by C_L proteins in the haemolymph. In resting insects, lipase is inhibited due to high concentration of free C_L proteins; but when lipoprotein A^+ is formed due to the action of AKH, concentration of C_L proteins in the haemolymph decreases and lipase becomes activated (Wheeler *et al*., 1986).

Other actions of AKH include stimulation of oxidation of substrates by flight muscles (Robinson and Goldsworthy, 1976, 1977; Goldsworthy, 1983), induction of cytochrome gene expression (Keeley, 1978; Keeley *et al*., 1991, 1996), myostimulation, especially cardiostimulation (O'Shea *et al*., 1984; Scarborough *et al*., 1984; Orchard, 1987; Gäde *et al*., 1997), inhibition of synthesis of proteins (Carlisle and Loughton, 1986) and RNA (Kodrik and Goldsworthy, 1995) in the fat body, stimulation of locomotory activity like

walking (Kodrik *et al*., 2000, 2002 a), induction of immune response (Goldsworthy *et al*., 2003) and regulation of reproduction (Gäde, 2004).

HORMONAL CONTROL OF LIPID MOBILIZATION AND RELEASE

The fat body is considered as a complex multifunctional and probably the most significant tissue in insects. The most obvious function of the fat body is the storage of reserve material (Locke, 1984; Dean *et al.*, 1985; Cotton and Anstee, 1991). The important food reserves of fat body are lipids, glycogen and proteins. In insects, lipid is the chief form in which energy is stored. Lipids form a very suitable energy source in insects since they occupy much less space for storage than an equivalent caloric quantity of protein and glycogen. This is of great importance for migratory and diapausing insects.

The major class of storage lipids of insect fat body is TAG (Walker *et al*., 1970; Chang and Friedman, 1971; Downer, 1985; Arrese and Wells, 1997; Canavoso *et al*., 1998). Fat body also contains small amounts of DAG and monoacylglycerols (Tietz, 1967; Walker *et al*., 1970; Beenakkers and Scheres, 1971). Other forms of lipids represented in insect fat body are sterols (Saito *et al.*, 1963; Gilbert and Goodfellow, 1965), phospholipids (Crone and Bridges, 1963; Fast, 1964) and free fatty acids (Nelson *et al.*, 1967; Beenakkers and Gilbert, 1968; Beenakkers and Scheres, 1971; Chang and Friedman, 1971).

The lipid stored in the insect fat body should be mobilized and released into the haemolymph to be transported to the site of metabolism. Fat body

lipolytic enzymes hydrolyse the stored TAG into DAG and fatty acids. The release form of lipids has been difficult to generalize, because it has been found that in different insects, it is either TAG (Martin 1969), DAG (Chino and Gilbert 1965; Beenakkers and Gilbert 1968; Downer and Steele, 1972; Thomas, 1974) or free fatty acids (Wlodawer and Lagwinska, 1967).

Under the influence of lipid mobilizing hormones, the TAG lipase gets activated and hydrolyses TAG into DAG primarily *sn*-1, 2-DAG. After the formation of DAG, it is moved from the fat droplet of cytosol to the plasma membrane possibly by a cytosolic carrier protein that binds to DAG and also interacts with the membrane, for delivering to the haemolymph (Arrese *et al*., 2001).

The DAG is translocated into the haemolymph and loaded into pre-existing HDLP (Arrese *et al*., 2001). During lipid release, HDLP binds to the lipophorin receptor on the plasma membrane. This binding of lipophorin has been shown to be Ca^{2+} dependent in the case of *M. sexta* (Tsuchida and Wells, 1990) but not in *L. migratoria* (Dantuma *et al*., 1996). The transfer of DAG from fat body to the HDLP is catalysed by a complex lipoprotein called lipid transfer particle (LTP) (Van Heusden and Law, 1989). This lipoprotein has been identified and purified from the haemolymph of several species of insects including *L. migratoria* (Hirayama and Chino, 1990), *M. domestica* (Capurro and De Bianchi, 1990), *P. americana* (Takeuchi and Chino, 1993), and *B. mori*

(Tsuchida *et al*., 1997). LTP has been found to be synthesized in the fat body and secreted into the haemolymph in *M. sexta* (Van Heusden *et al*., 1996).

Several hormones of the adipokinetic hormone group have been directly or indirectly involved in the mobilization and release of lipids from the fat body of insects. Besides, the biogenic amine, octopamine has also been found to be directly involved in lipid mobilization. Octopamine has been shown to induce a small but rapid and relatively long-lived hyperlipaemic response during stress in *Locusta* (Orchard *et al*., 1981). It plays a key role in insects that make long distance flight (Goldsworthy, 1983). In *S. gregaria*, octopamine stimulates the release of lipids during the first 10-20 min of flight (Orchard *et al*., 1982; Orchard and Lange, 1983 b). It mobilizes lipids in the house cricket, *A. domesticus* and adult *M. sexta* (Arrese *et al*., 1999) also. Van Heusden *et al*. (1984) reported that unlike AKH, octopamine induces the release of lipid without the formation of A^+ lipoprotein.

The role of AKH in the metabolism of lipids in insects has been well established, especially in the mobilisation and release of lipids from the fat body. Since its first demonstration by Mayer and Candy (1969) in *S. gregaria*, several insects belonging to various insect orders have been shown to possess similar hormones involved in similar regulatory processes. Both purified or synthetic AKH and extract of CC, which is the primary source of AKH, were found to have hyperlipaemic activity in several insect species especially in grasshoppers, lepidopterans, crickets and bugs (Beenakkers, 1969; Ziegler,

1979; Gäde, 1980, 1984, 1990 a, 2004; Herman and Dallmann, 1981; O'Shea *et al*., 1984; Gäde and Scheid, 1986; Ziegler *et al*., 1988; Woodring *et al*., 1989; Kumari and Gokuldas, 2001; Kodrik *et al*., 2002 a). Adipokinetic hormone can mobilize lipids from fat body of insects of almost all stages of life cycle and play a key role in mobilizing lipids during different locomotory actions like flight, walking and swimming and also during other activities of energy demand (Goldsworthy, 1983; Kodrik *et al*., 2002 a; Gäde, 2004).

SIGNAL TRANSDUCING MECHANISMS OF NEUROHORMONES

The peptide hormones and biogenic amines act as external signals and communicate with the target cell through specific receptors and intracellular second messengers by a mechanism referred to as signal transduction. Studies on signal transducing mechanisms of hormones in insects have attained greater importance nowadays (Berridge and Prince, 1975; Spencer and Candy, 1976; Candy, 1978; Putney, 1986 a; Van Marrewijk *et al*., 1993; Becker *et al*., 1998; Choi and Jurenka, 2004; Fellner *et al*., 2005).

Berridge (1980 a, b) reviewed on two separate pathways for signal transducing mechanisms of hormones. They are the cyclic AMP signalling system and calcium signalling system.

The cyclic AMP signalling system

The importance of cAMP in hormone action was first recognised by Sutherland and Rall (1958). Since then, this compound has been implicated in

cellular control mechanisms in a wide range of organisms from bacteria to mammals (Robison *et al*., 1968). The possible role of cAMP as an intracellular second messenger in the action of various insect hormones has been indicated by several workers. The hindgut stimulation by neurohormone produced by the brain of *L. maderae* (Marks *et al*., 1973; Holman and Marks, 1974; Nelson and Marks, 1980), cuticular tanning induced by bursicon in *P. americana* (Vandenberg and Mills, 1974), activity of diuretic hormone in *R. prolixus* (Aston, 1975), lipid mobilzation from the fat body by AKH in locusts (Spencer and Candy, 1976; Gäde and Holwerda, 1976; Stagg and Candy, 1996), hyperglycemic activity of CC extracts in *P. americana* (Hanaoka and Takahashi; 1977, Gäde, 1977; Ziegler *et al*., 1979), regulation of JH biosynthesis by CA by a factor present in the brain of *D. punctata* (Aucoin *et al*., 1987), activity of big PTTH (25.5 kDa) on the PG of pupal *M. sexta* (Smith *et al*., 1996) and the myotropic activity of *Acheta* diuretic peptide on the foregut of the house cricket, *A. domesticus* (Blake *et al.*, 1996) are some examples of hormonal activities of insects shown to be mediated by cAMP.

There are two definite phases of cAMP signalling system (Berridge, 1980 a). During the first phase, cyclic AMP is produced in the cell from ATP through a cascade of reactions involving the binding of hormone to the membrane receptor followed by the conformational changes of G protein resulting in the activation of adenyl/adenylyl/adenylate cyclase (AC) (Sutherland, 1972; Pfeuffer and Helmreich, 1975; Cassel and Selinger, 1978;

Lefkowitz *et al*., 1981; Gomperts *et al*., 2003). During the second phase, cAMP activates protein kinases which in turn phosphorylate various proteins involved in biochemical pathways (Berridge, 1980 a).

Calcium signalling system

Calcium ion is a well known intracellular second messenger controlling numerous vital processes (Bootman and Berridge, 1995). Ionised calcium (Ca^{2+}) is the most common signal transduction element in cells ranging from bacteria to specialized neurons (Clapham, 1995). Several workers suggested the role of calcium as second messenger in the signal transducing mechanism of insect hormones like PTTH (Smith and Gilbert, 1986), AKH, HTH (Van Marrewijk *et al*., 1993; Park and Keeley, 1996), PBAN (Matsumoto *et al*., 1995), diuretic peptide (Blake, *et al.*, 1996) and CCAP (Donini and Lange, 2002).

Berridge (1980 a) suggested the existence of three phases in the calcium dependent signal transduction. The first phase involves the release of Ca^{2+} from intracellular stores. The transducing mechanism responsible for releasing internal Ca^{2+} has been thoroughly studied during the past few years. The Ca^{2+} signalling system gets activated when the hormone binds to the specific G protein linked 7 transmembrane receptor (Berridge, 1993 a). The binding of the agonist to the receptor activates the enzyme, phospolipase C which brings about the hydrolysis of the minor plasma membrane phospholipid, phosphatidyl inositol 4,5-bisphosphate, to form IP_3 and DAG (Berridge, 1983).

The available evidence strongly indicates that IP_3 stimulates Ca^{2+} release from intracellular stores (Michell, 1975; Berridge, 1983). The organelle, from which IP_3 releases Ca^{2+}, was originally believed to be a component of ER. However, Volpe *et al.* (1988) suggested that the Ca^{2+} comes from the IP_3 responsive organelle, termed calciosome. The mechanism by which IP_3 releases Ca^{2+} appears to involve its interaction with a specific membrane receptor (Spat *et al*., 1986; Supattapone *et al*., 1988) called IP_3 receptor (IP_3R). Binding of IP_3 to its receptor increases Ca^{2+} efflux from the intracellular calciosome by opening a Ca^{2+} channel, which may be closely associated with the receptor (Ehrlich and Watras, 1988).

Calciosome was originally thought to be the IP_3-sensitive store, but later studies on Purkinje neurons suggest that these organelles may be heterogenous as some may be sensitive to ryanodine (Volpe *et al*., 1991). Thus at least two types of intracellular channels of calcium stores are identified; IP_3-sensitive calcium channel and ryanodine-sensitive calcium channel (Berridge, 1993 a, b). The distribution of such IP_3 or ryanodine sensitive calcium stores varies considerably from cell to cell. The calcium stores of some cells have either ryanodine sensitive receptor (RyR) or IP_3R, whereas others have both (Berridge, 1993 a). The former one is activated by the second messenger, cyclic ADP-ribose (cADPR) (Galione *et al*., 1991; Meszaros *et al*. 1993). But it remains to be seen, however, whether the level of cADPR changes in response to conventional extracellular signals such as hormones and neurotransmitters

(Berridge, 1993 b). Both IP_3R and RyR are sensitive to local Ca^{2+} concentration, so that a small increase in the cytosolic level of Ca^{2+} triggers an explosive release of stored calcium from IP_3- and ryanodine-sensitive calcium stores. This positive feed back process where by calcium triggers its own release is called calcium-induced calcium release (Gomperts, 2003).

There is an alternative suggestion that cAMP can affect intracellular calcium (Ca^{2+}_i) levels by influencing the intracellular pools of calcium contained in the mitochondria or sarcoplasmic reticulum (Rasmussen, 1970; Epstein *et al*., 1971). The studies of Berridge and Prince (1975) on the salivary glands of the adult blowfly *C. erythrocephala* reveals that cAMP acts as an intracellular messenger in the release of calcium from the intracellular pool.

In the second phase of calcium signalling cascade, the cytosolic free Ca^{2+} bind to the specific calcium binding protein molecules which act as internal receptors. The best known among such proteins is calmodulin (Gomperts *et al*., 2003). This molecule mediates the calcium induced reactions. The calcium-calmodulin complex activates or regulates a variety of enzymes in eukaryotic cells (for reviews, see Klee *et al*., 1980; Means *et al*., 1982). In insects calmodulin has been detected in various tissues (Cox *et al*., 1981; Dudoignon *et al*., 1983; Bodnaryk and Morishima, 1984; Morishima, 1987). Calcium/ calmodulin dependent cyclic nucleotide phosphodiesterase has been found in various sources. For example, head of *Drosophila* (Yamanaka and Kelly, 1981) and fat body of the silk moth, *B. mori* (Morishima *et al*., 1985).

Calmodulin stimulated AC has also been detected in different tissues from insects, like brain of the moth, *Mamestra configurata* (Bodnaryk, 1983) and fat body of the silk moth, *B. mori* (Morishima, 1984).

The third phase of calcium signalling system involves the entry of Ca^{2+}_{o} through the store operated channels of the plasma membrane (Gomperts *et al*., 2003). The Ca^{2+} influx is a consequence of IP_3-induced emptying of intracellular Ca^{2+} stores (Casteels and Droogmans, 1981; Putney, 1986 a). This interplay between calcium release and calcium entry has been termed capacitative calcium entry (Putney, 1986 a; Putney and Bird, 1993; Berridge, 1995).

There are two hypotheses regarding how the emptied ER stores give signal to open the plasma membrane Ca^{2+} channels (Clapham, 1995). The first supposes a direct physical link between the receptor for IP_3 and a putative surface Ca^{2+} channel; the second is that a diffusible second messenger named Ca^{2+} influx factor released from the ER opens Ca^{2+} channels of the plasma membrane (Randriamampita and Tsien, 1993; Clapham, 1994, 1995). The Ca^{2+} influx factor was originally proposed to be a low molecular mass (<500 Daltons) phosphorylated anionic compound that is stored in the ER awaiting the onset of store depletion for its release (Randriamampita and Tsien, 1993).

EXTRACELLULAR CALCIUM AND SIGNAL TRANSDUCTION

In eukaryotes including insects, the ionized calcium concentration in the extracellular fluids is maintained stably at a range of 1-2 mM (Levine and Williams, 1982; Dawson, 1990), at least 10,000 fold higher than the resting level of intracellular calcium concentration ($[Ca^{2+}]_i$) in most cells providing a seemingly inexhaustible supply of Ca^{2+} for its diverse intracellular functions (Brown *et al.*, 1995). Capacitative calcium entry is an inevitable part of calcium signalling system and many insect hormones activate the cell through calcium signalling system. Extracellular calcium is involved in the signal transduction of several insect hormones. However, the relative importance of $Ca^{2+}{}_o$ in signal transduction of any particular hormone appears to be different even in closely related insect species.

The importance of Ca^{2+} for the stimulation of glycogen phosphorylase and subsequent trehalose synthesis by hypertrehalosaemic factors of CC extract and HTH is well established in different insects. McClure and Steele (1981) found that *in vitro* activation of glycogen phosphorylase by purified CC extract, in intact fat body of *P. americana* was absolutely dependent on Ca^{2+} in the extracellular medium and omission of Ca^{2+} from the medium by chelation with 2 mM ethylene glycol-0,0'-bis(2-aminoethyl)-N,N,N'N'-tetraacetic acid (EGTA) significantly reduced the phosphorylase activity. Maximum activation of the enzyme was attained within 5 min by 1.8 mM calcium chloride and high concentration of Ca^{2+} was found inhibitory since the percentage of activation

was reduced with 5 mM calcium chloride. Orr *et al.* (1985) further demonstrated that in the presence of 1.8 mM calcium chloride, the purified hypertrehalosaemic peptides HT-I and HT-II increased the percentage of active phosphorylase in the fat body of *P. americana* by 2 to 3 fold. The activity, however, was found to be abolished by 2 mM EGTA. The presence of Ca^{2+}_{o} is also essential for the fat body from *P. americana* to synthesise and release trehalose in response to CC extracts and synthetic HT-I and the effect is either decreased or abolished when calcium is excluded by EGTA (Orr *et al*., 1985; Steele and Paul, 1985). The studies conducted with EGTA in dispersed trophocytes from the fat body of *P. americana* further supported the fact that Ca^{2+}_{o} is essential for the activation of glycogen phosphorylase by the synthetic HTH (Steele and Ireland, 1999).

Fat body of *L. migratoria* also requires Ca^{2+}_{o} for the *in vitro* stimulatory action of Lom-AKH-I on glycogen phosphorylase (Van Marrewijk *et al*., 1991). At low concentrations of Ca^{2+} (< 0.25 mM), AKH failed to activate the enzyme and a high level of Ca^{2+} of at least 1 mM, in the medium was needed for AKH to induce maximal enzyme activation. Vroemen *et al*. (1995 a) obtained similar results with Lom-AKH-I, -II and -III. But unlike *P. americana*, higher concentrations of Ca^{2+} (up to 4 mM) have no inhibitory effect in locusts (Van Marrewijk *et al*., 1991; Vroemen *et al.*, 1995 a).

In the oriental cockroach, *B. discoidalis*, Ca^{2+}_{o} found to have a minor role in modifying the hypertrehalosaemic action of Bld-HrTH (Lee and

Keeley, 1994; Keeley and Hesson, 1995; Keeley *et al.*, 1996). Even though the basal level of hormonal response is independent of Ca^{2+}_{o}, the presence of Ca^{2+}_{o} is essential to attain a maximal response (Keeley and Hesson, 1995). They also reported that the presence of Ca^{2+}_{o} is important only when the intracellular Ca^{2+} stores are depleted.

The importance of Ca^{2+}_{o} in the stimulation of fat body glycogen phosphorylase by the endogenous neuropeptide (Mem-CC) in the fruit beetle, *P. sinuata* has been studied by Auerswald and Gäde (2001 b). Maximum activation was achieved with 1.2 mM calcium which was, however, considerably suppressed by EGTA.

Relatively little is known about the role of Ca^{2+}_{o} in modulating the hyperlipaemic effect of AKH in insects. The first evidence for the involvement of Ca^{2+}_{o} in the AKH-induced mobilization of lipids from the insect fat body came from Spencer and Candy (1976) who reported a complete dependency of this lipid release on Ca^{2+}_{o} in *S. gregaria*. Similarly fat body of *L. migratoria* also requires Ca^{2+}_{o} for AKH-stimulated DAG production and its release into the haemolymph (Wang *et al*., 1990; Lum and Chino, 1990). By using extracellular calcium chelator, 1,2-bis(*o*-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid (BAPTA), Arrese *et al.* (1999) demonstrated that Ca^{2+}_{o} is essential for the AKH induced *in vivo* lipolysis and mobilization of lipids from the fat body of *M. sexta*.

Just like hypertrehalosaemic and hyperlipaemic effects of AKH on the insect fat body, the *in vitro* hyperprolinaemic effect of AKH is also regulated by Ca^{2+}_{o}. In *P. sinuata*, the transduction of hyperprolinaemic signal of Mem-CC requires Ca^{2+}_{o} (Auerswald and Gäde, 2001 a). Maximal proline synthesis was brought about by calcium levels higher than 0.5 mM.

In addition to the mobilization of food reserves of fat body, most of the other hormonal actions of the members of AKH/RPCH family are also regulated by Ca^{2+}_{o}. Evidence for the influence of Ca^{2+}_{o} on the inhibition of lipid synthesis induced by AKH has been obtained by Gokuldas (1989) from his studies with the fat body of *S. gregaria*. He demonstrated that AKH inhibits acetate incorporation into lipids in the presence of Ca^{2+}_{o} and the hormone has no significant inhibitory effect in the absence of Ca^{2+}. In the adult male locust, *L. migratoria*, none of the AKH was able to elevate fat body cAMP levels in the absence of Ca^{2+}, while in the presence of 1.5 mM Ca^{2+}, intracellular cAMP levels were raised significantly by the three AKH (Vroemen *et al*., 1995 a). The CC extracts and synthetic Lom-AKH-I, -II and -III inhibited total RNA synthesis in the fat body of *L. migratoria* and the inhibition was not seen when fat bodies from male locusts were incubated in Ca^{2+}-free medium containing EGTA (Kodrik and Goldsworthy, 1995). Physiological level of Ca^{2+}_{o} is important for transducing the Bld-HrTH message for cytochrome P4504C1 gene (*CYP4C1)* expression in *B. discoidalis* (Keeley *et al*., 1996). Sun and Steele (2002) demonstrated that in dispersed fat

body of the cockroach, *P. americana*, HTH-II required Ca^{2+}_{o} for the activation of phospholipid hydrolyzing enzyme, phospholipase A_2 (PLA_2). The HTH-II failed to activate PLA_2 when Ca^{2+} in the medium was chelated with EGTA.

Ecdysteroid secretion by insect PG induced by PTTH was shown to be Ca^{2+} dose-dependent in *M. sexta*. There was a dramatic increase of PTTH activity when calcium concentration was increased from 0.1 mM to 10 mM (Smith and Gilbert, 1986). The PTTH-induced adenylate cyclase activation and steroidogenesis in *M. sexta* has been found to get inhibited by an omission of Ca^{2+} from the incubation medium with or without EGTA. Similar dose-dependent effect of Ca^{2+} on ecdysteroid secretion was noticed in the larvae of *B. mori*. Here, 1 mM Ca^{2+} was sufficient to elicit the response of PG to PTTH and 10 mM Ca^{2+} induced very high response (Gu *et al*., 1998). Dependence of PTTH action on Ca^{2+}_{o} in *M. sexta* was also reported by Fellner *et al*. (2005) and Priester and Smith (2005).

In *D. punctata*, the inhibitory effect of allatostatin (brain extract) on JH release from CA was influenced by Ca^{2+} in the medium. High inhibition obtained (75%) with low calcium concentration (1.3 mM) was reduced and abolished when Ca^{2+} concentration was increased to 5 and 10 mM respectively (Aucoin *et al*., 1987). In *Mythimna loreyi*, the allatotropic activity of both the sub-oesophageal ganglion and CC extract on the CA were dependent on Ca^{2+} concentration in the medium (Kou and Chen, 2000 a, b).

PBAN-stimulated sex pheromone production is dependent on Ca^{2+}_{o} in the red banded leaf roller moth, *Argyrotaenia velutinana* (Jurenka *et al*., 1994), the European corn borer *Ostrinia nubilalis* (Ma and Roelofs, 1995), the American bollworm, *Helicoverpa armigera* (Rafaeli and Gileadi, 1996), the pine caterpillar moth, *Dentrolimus punctatus* (Zhao *et al*., 2002) and the corn earworm, *H. zea* (Choi and Jurenka, 2004). In *H. armigera* pheromonotropic effect of PBAN could only be seen in the presence of 1 to 5 mM calcium and no activity was noticed either in the absence or at high levels of calcium in the extracellular medium (Rafaeli and Gileadi, 1996).

Lipid synthesis inhibition and lipid mobilization triggered by octopamine have been found to require a physiological range of Ca^{2+}_{o} in *G. morsitans*, and the effect was abolished when Ca^{2+} in the medium was chelated with EGTA (Pimley, 1985). In *L. migratoria*, octopamine-mediated AKH release from the glandular lobes of CC was found to be fully dependent upon the presence of Ca^{2+}_{o}. With a concentration of 4 mM calcium chloride, octopamine released sufficient AKH from CC to produce an approximately 100% increase in lipid levels (Pannabecker and Orchard, 1987). Extracellular Ca^{2+} is also important for octopamine dependent phosphorylase activation in the fat body of *B. discoidalis* (Park and Keeley, 1995).

The need for Ca^{2+} for the action of proctolin on the hindgut was first demonstrated in *L. maderae* (Cook *et al*., 1975). Calcium also intervenes in the proctolin-induced contractions of extensor tibial (Evans, 1984 a, b) and

oviduct muscles of *L. migratoria* (Lange *et al*., 1987) and heart (Shukle and Judson, 1984) and hyperneural muscle of *Periplaneta* (Hertel and Penzlin, 1986). In the silkworm, eclosion hormone-induced activation of nitric oxide synthase and cGMP production followed by triggering of ecdysis behaviour was dependent on calcium (Shibanaka *et al*., 1994). The myotropic activity of *Acheta* diuretic peptide on the foregut of the house cricket, *A. domesticus*, is also dependent on $Ca^{2+}{}_o$ (Blake *et al*., 1996). The peptide CAP_{2b} which stimulates fluid transport in *Drosophila* MT is dependent on $Ca^{2+}{}_o$ (MacPherson *et al*., 2001). Similarly the CCAP-induced contractions of oviducts in *L. migratoria* are dependent on $Ca^{2+}{}_o$ (Donini and Lange, 2002).

For the signal transduction, insect neurohormones depend on $Ca^{2+}{}_o$ mostly for increasing the cytosolic concentration of Ca^{2+} ($[Ca^{2+}]_i$) and an increase in $[Ca^{2+}]_i$ is a prerequisite for the signal transduction. Several workers used radioactive Ca^{2+}, $^{45}Ca^{2+}$ or calcium sensing fluorescent dyes to trace the movement and time course of concentration of calcium following a challenge with the hormone. The studies carried out with $^{45}Ca^{2+}$ illustrated that hypertrehalosaemic agents of CC induce the uptake of Ca^{2+} by fat body cells in *P. americana* (Steele and Paul, 1985). The concept of hormone-induced calcium influx was proved to be valid for Lom-AKH-I, -II and III (Wang *et al.*, 1990; Van Marrewijk *et al.*, 1993; Vroemen *et al*., 1995 a). Similarly, in *P. americana* and *P. sinuata*, studies using $^{45}Ca^{2+}$ have shown that, their

neuropeptide hormones enhanced calcium uptake from 30 to 100% (Steele and Ireland, 1999; Auerswald and Gäde, 2001 b).

First direct evidence to show that $[Ca^{2+}]_i$ is increased by hormone treatment came from the studies of Jahagirdar *et al*. (1987). The intensity of fura-2 fluorescence of cultured hemocytes of *Malacosoma disstria* increased following treatment with HTH-I and HTH-II. Similarly, NHD- and HTH-induced Ca^{2+} influxes were demonstrated in *P. americana* (Wicher and Reuter, 1993; Sun and Steele, 2001). Such Ca^{2+} influxes were also induced by PTTH in *Galleria mellonella* (Birkenbeil, 1996), *M. sexta*, (Birkenbeil, 1998; Fellner *et al*., 2005) and *B. mori* (Dedos *et al*., 2005).

The importance of $Ca^{2+}{}_o$ influx in the signal transduction of the insect hormones was evaluated using the calcium ionophore, A23187. This antibiotic facilitates $Ca^{2+}{}_o$ transport across plasma membranes (Reed and Lardy, 1972; Pfeiffer *et al*., 1978) and thereby mimics the action of the hormone.

A23187-induced activation of glycogen phosphorylase in the presence of Ca^{2+} was found to mimic the activating effect of hypertrehalosaemic factors in different insects such as *P. americana*, *L. migratoria*, *P. sinuata* and *B. discoidalis* (McClure and Steele, 1981; Van Marrewijk *et al*., 1991; Keeley *et al*., 1995; Park and Keeley, 1996; Steele and Ireland, 1999; Auerswald and Gäde, 2001 b). This suggests that the influx of Ca^{2+} is essential for the activation of enzyme by the hypertrehalosaemic factors in insects. In *S. gregaria* AKH-mediated inhibition of acetate incorporation into the fat body

lipids was mimicked by A23187 in the presence of Ca^{2+} (Gokuldas, 1989). Similarly, the hyperprolinaemic effect of Mem-CC was found to be mimicked by A23187 in *P. sinuata* (Auerswald and Gäde, 2001 a).

The importance of the influx of $Ca^{2+}{}_{o}$ into the target cells in modulating the PTTH-mediated steroidogenic effect was demonstrated using A23187 in the pupa (Smith *et al*., 1985; Smith and Gilbert, 1986) and the larva (Hayes *et al*. 1995) of *M. sexta* and in *B. mori* (Gu *et al*., 1998).

A23187 mediates at least part of the effects of octopamine which inhibits lipid synthesis in *G. morsitans* (Pimley, 1985). Similar inward movement of Ca^{2+} assisted by A23187 resulted in the release of AKH from locust CC, as in the case of octopamine induced release of AKH from CC. (Pannabecker and Orchard, 1987). In *D. punctata*, A23187 elicited flux of calcium across cell membranes and caused a rapid and irreversible decline in JH release by the CA (Kikukawa *et al*., 1987). In *O. nubilalis*, A23187 mimicked the action of PBAN at a concentration of 10 µM (Ma and Roelofs, 1995).

The significance of hormone-induced influx of $Ca^{2+}{}_{o}$ has further been investigated in insects using calcium channel antagonists such as lanthanum (La^{3+}), cadmium (Cd^{2+}) and cobalt (Co^{2+}). Lanthanum ions (La^{3+}) can be used to block plasma membrane calcium channels so as to prevent Ca^{2+} entry (Weiss, 1974, Fitzpatrick, 1990) and it does not permeate cell membranes (Henrikson, 1974; Szasz *et al*., 1978). Cadmium and cobalt can act as low

affinity Ca^{2+} channel blockers and can block non-specific calcium channels of plasma membranes.

The *in vitro* studies conducted in the fat body of the cockroaches, *P. americana* (McClure and Steele, 1981) and *B. discoidalis* (Keeley and Hesson, 1995; Park and Keeley, 1996) and the locust, *L. migratoria* (Van Marrewijk *et al*., 1992) showed that the activation of glycogen phosphorylase by the hypertrehalosaemic factors has been blocked by La^{3+}. Lanthanum has also been found to suppress Bld-HrTH-induced mRNA synthesis in *B. discoidalis* (Keeley *et al*., 1996).

The efficacy of metallic ions as calcium channel blockers was demonstrated with regard to PTTH-stimulated Ca^{2+} influx and subsequent physiological actions. PTTH-stimulated ecdysone synthesis was greatly suppressed by La^{3+} in *M. sexta* (Smith *et al.*, 1985; Hayes *et al*., 1995) and in *B. mori* (Gu *et al*., 1998). Birkenbeil (1998) demonstrated that PTTH-induced increase in $Ca^{2+}{}_{i}$ levels of PG of *M. sexta* was totally inhibited by 1 mM cadmium whereas nickel and lanthanum only partially reduced the influx. In the PG cells of *M. sexta*, both La^{3+} and Cd^{2+} could block PTTH stimulated Ca^{2+} influx and the consequent extracellular signal regulated kinase phosphorylation (Rybczynski and Gilbert, 2003).

The TKYFSPRLamide-induced pheromone production was blocked by 10 mM $LaCl_3$ in the common cut worm, *Spodoptera litura* (Matsumoto *et al*., 1995). In locust, La^{3+} inhibits Ca^{2+} transport across plasma membrane which

in turn prevents octopamine mediated cAMP production followed by AKH release (Pannabecker and Orchard, 1987). The presence of 0.1 mM Cd^{2+}, in normal saline, prevented NHD-induced increase of $[Ca^{2+}]_i$ of dorsal midline neurons of *P. americana* (Wicher and Reuter, 1993). Hsieh *et al*. (2003) demonstrated that in *M. loreyi*, $Ca^{2+}{}_o$-induced JHA release was significantly reduced by metallic ions with an efficacy sequence of $Cd^{2+} > La^{3+} > Co^{2+}$. But in *H. zea*, La^{3+} completely blocked PBAN-induced pheromone production (Choi and Jurenka, 2004).

Insect hormones that belong to AKH/RPCH family depend on $Ca^{2+}{}_o$ also for their binding to the receptor. Ziegler *et al*. (1995) reported that $Ca^{2+}{}_o$ is absolutely necessary for binding AKH to the respective receptor on the fat body cells of *M. sexta*. A possible involvement of $Ca^{2+}{}_o$ was suggested for the binding of Lom-AKH-I, -II and -III to their respective receptors in locust fat body (Van der Horst *et al*., 1997; Vroemen *et al*., 1998). Extracellular Ca^{2+} is also important for binding of the hormone, Mem-CC to the receptor in *P. sinuata* (Auerswald and Gäde, 2001 b).

Besides the aforesaid role of $Ca^{2+}{}_o$ in mediating several hormone-induced activations of cellular enzymes leading to various physiological actions, $Ca^{2+}{}_o$ alone has been found to initiate several such activities even in the absence of any signal molecule. For example, $Ca^{2+}{}_o$ induces activation of glycogen phosphorylase in *P. americana* (McClure and Steele, 1981) and *B. discoidalis* (Steele and Paul, 1985; Park and Keeley, 1995), mobilization and

release of lipids from the fat body in *S. gregaria* (Spencer and Candy, 1976) and JH release in *D. punctata* (Kikukawa *et al*., 1987), *L. migratoria* (Dale and Tobe, 1988), *M. sexta* (Allen *et al*., 1992), *G. bimaculatus* (Klein *et al*., 1993) and *M. loreyi* (Hsieh *et al*., 2002).

INTRACELLULAR CALCIUM AND SIGNAL TRANSDUCTION

The concentration of cytosolic free calcium undergoes rapid and often substantial fluctuation in response to the binding of extracellular first messengers to their cognate receptors on target cells (Brown *et al*., 1995). This cytosolic free calcium comes either from the intracellular calcium stores or from extracellular space (Putney, 1978, 1986 a, b; Putney *et al*., 1981; Takemura *et al*., 1989; Simpson *et al*., 1995; Berridge, 1997). Calcium from intracellular reserves can be mobilized either by hormones or by other agents like thapsigargin or thimerosal. The thapsigargin inhibits the ATP dependent Ca^{2+} pump of ER and triggers release of Ca^{2+} from IP_3-insensitive stores (Thastrup *et al*., 1990), whereas thimerosal sensitises IP_3 receptors of ER by thioalkylation for Ca^{2+} release (Bootman *et al*., 1992). But Ca^{2+} antagonists like dantrolene, ryanodine and TMB-8 inhibit the release of $Ca^{2+}{}_i$ from calcium stores (Lydan and O'Day, 1988; Ohta *et al*., 1990; Meissner, 1994) after a hormone challenge.

In addition to the influx of $Ca^{2+}{}_o$, the mobilization of Ca^{2+} from intracellular stores is also important in the signal transduction of many insect neurohormones. The involvement of $Ca^{2+}{}_i$ derived from Ca^{2+} stores in the

action of hypertrehalosaemic peptides was investigated in different insects. The dependence of glycogen phosphorylase activation by AKH on Ca^{2+} mobilized from calcium stores was established in *L. migratoria* (Van Marrewijk *et al*., 1993) and *B. discoidalis* (Keeley and Hesson, 1995). Moreover, calcium mobilization by thimerosal and thapsigargin could mimic the hypertrehalosaemic effects of Bld-HrTH in *B. discoidalis* to a great extent which reflects that $Ca^{2+}{}_i$ derived from both IP_3-dependent and IP_3-independent calcium stores are involved in hormonal action of Bld-HrTH (Keeley and Hesson, 1995; Park and Keeley, 1996; Keeley *et al*., 1996).

In *P. sinuata,* the Mem-CC-modulated trehalose synthesis was reduced from 54% to 44% when $Ca^{2+}{}_i$ release was blocked with dantrolene. But thimerosal (0.1 mM and 0.2 mM) and thapsigargin (1 mM) could activate fat body phosphorylase significantly above control and this activation was almost identical to that achieved by the neurohormone, Mem-CC. These results indicate that $Ca^{2+}{}_i$ release is important in signal transduction of Mem-CC also (Auerswald and Gäde, 2001 a). Dependence of HTH on $Ca^{2+}{}_i$ was also established in the fat body trophocytes of *P. americana* where the chelation of $Ca^{2+}{}_i$, mobilized by Pea-HTH-I or -II, with Quin-2, Ca^{2+} chelator, resulted in 50% decrease of phosphorylase *a*, as generated by Pea-HTH-I or -II (Steele and Ireland, 1999). Similarly, the hypertrehalosaemic effect of HTH of *P. americana* was blocked by the incorporation of an intracellular Ca^{2+} chelator, 1,2-bis(*o*-aminophenoxy)ethane-N,N,N’N’-tetraacetic acid, acetoxymethyl

ester (BAPTA-AM) (Sun and Steele, 2002). Additional evidence supporting the hypothesis that elevated Ca^{2+}_i is required for the signal transduction of Pea-HTH-II was obtained by treating intact fat body of *P. americana* with thapsigargin which resulted in significant increase in trehalose efflux (Sun *et al*., 2002).

The importance of Ca^{2+}_i for *CYP4C1* gene expression in *B. discoidalis* was evaluated by injecting thimerosal into experimental animals. The result showed that, 100 µM thimerosal was as effective as a maximally stimulatory dose of Bld-HrTH for promoting *CYP4C1* expression. This indicates that Ca^{2+}_i from IP_3-sensitive Ca^{2+} store is important for the Bld-HrTH induced gene expression (Keeley *et al*., 1996). The requirement of Ca^{2+}_i for HTH-modulated activation of PLA_2 was demonstrated with BAPTA-AM and ryanodine. The chelation of Ca^{2+}_i with BAPTA-AM blocked the activation of PLA_2 by Pea-HTH-II. The increase in $[Ca^{2+}]_i$, induced by HTH was completely blocked by preincubation of the cells with 20 µM ryanodine and the same concentration of ryanodine also blocked activation of membrane PLA_2 by HTH (Sun and Steele, 2002).

The role of Ca^{2+} originating from intracellular sources in adipokinetic signalling is not thoroughly investigated so far and the knowledge is fragmentary. In locust fat body, during AKH action, intracellular release of Ca^{2+} takes place and leads to capacitative entry of Ca^{2+} (Van Marrewijk *et al*., 1993; Vroemen *et al*., 1995 a). In *M. sexta*, thapsigargin and ionomycin cause

increase in lipid release into the haemolymph demonstrating that Ca^{2+} from IP_3-insensitive intracellular stores is a part of adipokinetic signalling process (Arrese *et al*., 1999). Van der Horst (2003), however, suggested a possible involvement of Ca^{2+} from IP_3-sensitive Ca^{2+} store in lipid mobilization from the fat body of locusts.

Intracellular Ca^{2+} is involved in the signal transduction of PTTH also. By using thapsigargin, Gu *et al*. (1998) demonstrated that PTTH-stimulated ecdysone synthesis in *B. mori* is dependent on Ca^{2+}_i released from IP_3-insensitive Ca^{2+} stores. Similar mediation of PTTH action was also reported in the PG of *Manduca* larva (Birkenbeil, 1998). But Fellner *et al*. (2005) have shown that Ca^{2+} from IP_3-sensitive stores mediate PTTH triggered ecdysteroidogenesis in *M. sexta*.

Studies carried out with thimerosal and thapsigargin by Auerswald and Gäde (2001 a) showed that Ca^{2+} from IP_3-insensitive intracellular Ca^{2+} stores is involved in hyperprolinaemic action of Mem-CC in *P. sinuata*. But in the case of the myotropic peptide proctolin, the hormone promotes an increase in Ca^{2+}_i levels, in part, through the production of IP_3 in a number of insect muscle tissues (Lange *et al*., 1987; Lange, 1988; Baines *et al*., 1990; Wilcox and Lange, 1995; Mazzocco-Manneval *et al*., 1998; Wegener and Nässel, 2000). Antidiuretic activity of neuroparsins of *L. migratoria* is also dependent on Ca^{2+}_i from IP_3-sensitive calcium stores (Gäde *et al*., 1997). The diuretic activity of insect kinins in *Aedes aegypti* and *D. melanogaster* is also mediated

by Ca^{2+}_{i} from IP_3 dependent calcium stores (Cady and Hagedorn, 1999; Pollock *et al*., 2003). Similarly Manse-CAP_{2b} stimulated secretion by MT of *D. melanogaster* requires Ca^{2+} from IP_3-sensitive stores (Coast *et al*., 2002; Pollock *et al*., 2003). Intracellular Ca^{2+} is also involved in the CCAP activated oviduct contraction in locusts (Donini and Lange, 2002). But the exact nature of the Ca^{2+} store is not fully established.

HYPERLIPAEMIC SIGNAL TRANSDUCTION OF AKH

The hyperlipaemic signal transduction of AKH has been investigated only in a few insects (See Reviews by Arrese *et al*., 2001; Gäde and Auerswald, 2003; Van der Horst, 2003; Gäde, 2004). The model of adipokinetic signalling is based mainly on the studies on the locusts, *L. migratoria* and *S. gregaria* and the moth *M. sexta*.

The available data suggest that binding of the AKH to their plasma membrane receptor(s) at the fat body cells is the primary step to the induction of signal transduction events that ultimately lead to the mobilization of lipids (Wang *et al*., 1990; Zeigler *et al*., 1995; Van der Horst, 2003). The binding of AKH to the receptor is a Ca^{2+} dependent process (Zeigler *et al*., 1995). The first insect AKH receptors, identified at the molecular level have been in the fruit fly, *D. melanogaster* and the silk worm, *B. mori* (Staubli *et al*., 2002). The AKH receptor is found to be a G_s protein coupled receptor (Arrese *et al*., 1999) which mediates the signal transfer to the interior of the cell. In locusts and moths, the binding of AKH to the receptor leads to a conformational

change of G_s protein which in turn activates AC resulting in an increase in the intracellular cAMP levels (Gäde and Auerswald, 2003; Gäde, 2004).

AKH binding to the receptor causes an entry of Ca^{2+} via calcium channels into the fat body (Van Marrewijk *et al*., 1991; Vroemen *et al*., 1995 b; Gäde and Auerswald, 2003). These processes give rise to two intracellular second messengers, Ca^{2+} and cAMP (Gäde and Holwerda, 1976; Spencer and Candy, 1976; Lum and Chino, 1990; Wang *et al*., 1990; Arrese *et al*., 1999). The first evidence for the involvement of cAMP and Ca^{2+} in the AKH induced mobilization of lipids was from the fat body of *S. gregaria* (Spencer and Candy, 1976). The cAMP activates a protein kinase (Spencer and Candy, 1976) in the presence of Ca^{2+} (Pines *et al*., 1981; Wang *et al*., 1990) which in turn phosphorylates and thereby activates the lipolytic enzyme TAG lipase (Arrese *et al*., 1999). The presence of cAMP-dependent protein kinase A has been demonstrated in the fat body of both *L. migratoria* and *M. sexta* (Van Marrewijk *et al*., 1980; Arrese *et al*., 1999).

The possible involvement of IP_3 in the hyperlipaemic signal transduction of AKH is yet to be clarified. AKH increases levels of IP_3 in the fat body of the locusts, *S. gregaria* (Pancholi *et al*., 1991; Stagg and Candy, 1996) and *L. migratoria* (Van Marrewijk *et al*., 1996; Vroemen *et al*., 1998). Further, Vroemen *et al*. (1998) reported that AKH-I give rise to more IP_3 than AKH-II and the first hormone might serve predominantly as a lipid mobilizing hormone, while AKH-II may be the main trigger for carbohydrate

mobilization. These ideas indicate that IP_3 serve as an important second messenger for lipid mobilization from the fat body. This IP_3 may mediate the release of Ca^{2+} from intracellular stores and the depletion Ca^{2+} from intracellular stores stimulates the influx of $Ca^{2+}{}_o$, indicative of the operation of a capacitative (store operated) calcium entry mechanism (Van der Horst, 2003). However, Gäde and Auerswald (2002, 2003) found that IP_3 is not at all involved in the AKH-induced lipid mobilization in locusts. But $Ca^{2+}{}_i$ from IP_3-independent stores is involved in the activation of TAG lipase (Arrese *et al*., 1999; Gäde, 2004).

In the locust fat body, intracellular release of Ca^{2+} takes place and leads to a 'capacitative entry' of Ca^{2+} (Van Marrewijk *et al*., 1993; Vroemen *et al*., 1995 b), but the effects of these events in lipid mobilization is not fully understood (Gäde and Auerswald, 2003).

The foregoing review has dealt with the chemical structure, functions and signal transduction of insect hormones, especially the adipokinetic hormone. Calcium ion appears to be a major second messenger in the signal transduction of several insect neurohormones. Both extra- and intracellular Ca^{2+} seem to play key roles in Ca^{2+}-mediated hormonal actions. Levels of both $Ca^{2+}{}_o$ and $Ca^{2+}{}_i$ are found to modulate the AKH-mediated mobilization of food reserves and release of trehalose, DAG and proline in flying and active insects. Most of the works carried out in this area are confined to flying insects characterized by sustained activities and are yet to be explored in flightless

insects. Thus it was thought worthwhile to study the modulating effect of both Ca^{2+}_{o} and Ca^{2+}_{i} on the action of insect neuropeptide hormones in a flightless, sluggish insect, the plant bug, *I. limbata*.

In *I. limbata*, the hyperlipaemic activity of native peptides (crude CC extract) and cross reactivity of the heterologous hormone, Lom-AKH-I were demonstrated (Rasheed and Gokuldas, 2002). The present project was aimed to examine the chemical nature of the adipokinetic factor(s) of the crude extract of brain-CC-CA complex (crude CC extract) of *I. limbata* and to study the relative importance of both Ca^{2+}_{o} and Ca^{2+}_{i} in modulating Lom-AKH-I- and crude CC extract-mediated mobilization and release of lipids from the fat body of *I. limbata* using agonists and antagonists of calcium. The possible involvement of Ca^{2+}, cAMP and IP_3 as the second messengers of insect neuropeptide-induced lipid mobilization and release from the fat body of *I. limbata* are discussed.

Chapter 3

Materials and Methods

MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Experimental insects

Investigations were carried out in *Iphita limbata* Stål (Pyrrhocoridae: Heteroptera), a polyphagous bug. These are medium sized bugs with brick red colour and black markings. The size usually ranges from 19-21 mm (females) and 16-18 mm (males).

3.1.2. Chemicals

1. A23187 (Calcium ionophore)
2. Acetonitrile - HPLC grade
3. Bovine Serum Albumin - fraction V powder (BSA)
4. Calcium chloride
5. Cadmium chloride
6. Chloroform
7. Concentrated sulphuric acid
8. Dimethyl sulfoxide (DMSO)
9. Di-potassium hydrogen orthophosphate
10. EGTA (Ethylene glycol-0,0'-bis (2-aminoethyl) N,N,N'N'-tetraacetic acid) ($C_{14}H_{24}N_2O_{10}$)
11. Glycerol trioleate

12. Glucose

13. Lanthanum chloride

14. Magnesium sulphate

15. Methyl alcohol

16. N-2- Hydroxyethyl piperazin N-2 ethanesulphonic acid (HEPES)

17. Orthophosphoric acid

18. Potassium chloride

19. Sodium chloride

20. Sucrose ($C_{12}H_{22}O_{11}$)

21. Synthetic *Locusta migratoria* Adipokinetic hormone-I (Lom-AKH-I)

22. Trifluoroacetic acid (TFA) - HPLC grade

23. Thimerosal (Ethylmercurithiosalicylic acid) ($C_9H_9HgO_2SNa$)

24. Vanillin (4-Hydroxy-3-methoxy benzaldehyde)

Lom-AKH-I was purchased from Peninsula Laboratories (USA) and GenScript Corp. (USA). A23187 was obtained from Calbiochem (La Jolla, CA, USA) and thimerosal from Sigma (USA). Other chemicals were of analytical grade, and were obtained from local suppliers.

3.1.3. Equipments

1. Clinical centrifuge (Remi Equipments)

2. Centrifuge (refrigerated) (Plastro Crafts)

3. Desiccator (Borosil)

4. Deep freezer, -4 °C (Labline, India)

5. Deep freezer, -20 °C (Labline, India)

6. Freeze drier (Savant)

7. Hot air oven (KEMI)

8. High performance liquid chromatography (Shimadzu)

9. Microfuge (Beckman)

10. Magnetic stirrer (Toshnival)

11. Microlitre syringes (Hamilton)

12. Micropipettes (Lab Systems, Accupipet and Microlit)

13. pH Meter (GeNei, Bangalore Genei Ltd)

14. Refrigerator (Kelvinator and LG)

15. Stereo microscope (Carl Zeiss India Pvt. Limited)

16. UV-VIS Spectrophotometer (Shimadzu)

17. Ultrasonic liquid processor (Sonics & Materials INC, USA)

18. Water bath incubator shaker (Toshnival)

3.2. METHODS

3.2.1. Maintenance of stock culture of *I. limbata*

Adult insects were collected from their natural habitat and were transferred into 1 L glass jars containing green gram (*Phaseolus aureus*)

maintained on washed and sterilized sand. Approximately 20 insects were kept in each glass jar. The jars were covered with cotton cloth and kept in large insect cages protected from ants by water barrier. The stock culture was maintained at room temperature (28 ± 2 °C) under a 12 h : 12 h photoperiod regime in the insectary. Dead insects were removed from the jars every day to maintain the hygienic condition. Food and water were provided *ad libitum* and were replenished thrice a week. Mature adult insects of both sexes, after keeping at least for three days in the insectary, were used for the experiments.

3.2.2 Preparation of buffers, reagents and test solutions

3.2.2.1. A23187 solution

A stock solution of A23187 (1 mM) was prepared by dissolving 1.0 mg of the same in 1.910 ml of DMSO (100%). From this stock, solutions of different concentrations of A23187 were prepared by dilution with appropriate volumes of DMSO to get final concentrations of 1, 5, 10, 15, 20 and 25 µM, when 5 µl of the ionophore was added to the incubation buffer. The solutions were protected from light and water contamination and kept at -20 °C in a deep freezer.

3.2.2.2. Calcium chloride solution

A stock solution of 80 mM $CaCl_2$ was prepared by dissolving 1.176 g $CaCl_2$ in 100 ml double distilled water. From this stock solution, $CaCl_2$ solutions of different concentrations were prepared by dilution with distilled

water, so as to obtain final concentrations of 8.0, 4.0, 2.0, 1.5 and 1.0 mM, when 50 µl of these calcium chloride solutions were added to 200 µl of HEPES buffer or 15 µl were injected into (approx.) 60 µl of haemolymph of the body cavity.

3.2.2.3. Cadmium chloride solution

Cadmium chloride (366.6 mg) was dissolved in 100 ml of distilled water. This solution (20 mM) served as the stock solution. From this, different concentrations of $CdCl_2$ were prepared so as to get final concentrations of 0.1, 0.5, 1.0, 1.5 or 2.0 mM when 50 µl each of the solution was added to the incubation medium.

3.2.2.4. Chloroform-methanol mixture

Chloroform and methanol were mixed in the ratio 1:2 (v/v). The mixture was stored well stoppered.

3.2.2.5. EGTA solution

A working solution of 22 mM EGTA was prepared by dissolving 83.677 mg of EGTA in 10 ml of 0.5 N NaOH solution. Final concentration of EGTA was maintained at 2 mM in the incubation medium.

3.2.2.6. HEPES buffer

The medium used for fat body *in vitro* incubation was HEPES buffer with or without $CaCl_2$. Calcium chloride solutions of various concentrations

were prepared separately and mixed with incubation buffer lacking $CaCl_2$, wherever necessary. The HEPES buffer (with $CaCl_2$) contained 10 mM NaCl, 12 mM KCl, 2 mM $MgSO_4$, 1 mM K_2HPO_4, 1 mM $CaCl_2$, 30 mM HEPES, 10 mM glucose, 50 mM sucrose and 2% (w/v) BSA. The ingredients were dissolved in distilled water and the pH of the buffer was adjusted to 7.2. The buffer was kept frozen and thawed just before experiment.

3.2.2.7. Lanthanum chloride solution

Lanthanum chloride (742.74 mg) was dissolved in double distilled water (100 ml) to get a stock solution of 20 mM. From this stock, solutions with different concentrations were prepared by dilution with double distilled water so as to obtain final concentrations of 0.1, 0.5, 1.0, 1.5 or 2.0 mM of $LaCl_3$, when 50 µl of the solution was added to 200 µl of the HEPES buffer.

3.2.2.8. Lipid standard solutions

Lipid standard stock solution was prepared by dissolving 500 mg of chromatographically pure glycerol trioleate in 50 ml of chloroform. A working standard solution was prepared by diluting 1 ml of the stock solution to 10 ml with chloroform so that 1 µl of the solution contained 1 µg of lipid. Appropriate volumes of this solution were taken for the purpose of calibration and also for quantitation of lipid samples in various experiments.

3.2.2.9. Lom-AKH-I solution

A stock solution of 100 µmol of Lom-AKH-I was prepared by dissolving 1 mg of Lom-AKH-I in 8.625 ml of 80% methanol. From this stock solution, 1 ml was transferred to a glass vial and the solvent was removed by evaporation in a desiccator. The dried material was then dissolved in 10 ml double distilled water to get a solution of 10 µmol. From this, working solutions of 50 pmol, 100 pmol and 200 pmol /10 µl of Lom-AKH-I were prepared in double distilled water.

3.2.2.10. Phosphovanillin reagent

The reagent, used for the spectrophotometric analysis of lipids, was prepared by mixing concentrated orthophosphoric acid and 0.525% (w/v) aqueous vanillin in the ratio 3:2 (v/v).

3.2.2.11. Sodium chloride solution

Sodium chloride solution (1 M) was prepared by dissolving 5.844 g of the salt in 100 ml double distilled water.

3.2.2.12. Thimerosal solution

A stock solution of 8.8 mM thimerosal was prepared by dissolving 35.62 mg of thimerosal in 10 ml of double distilled water. From this, various concentrations of thimerosal were prepared by dilution with distilled water to get final concentrations of 25, 50, 100, 150 or 200 µM when 20 µl of the solution was added to the incubation buffer (200 µl).

3.2.3. Preparation of crude extract of brain-CC-CA complex of *I. limbata* for RP-HPLC fractionation

A crude extract of brain-CC-CA complex of *I. limbata* was prepared by a modified method of that used by Gäde (1994). A superficial incision was made on the dorsal surface of the head capsule of the plant bug. The incision was broadened and the brain-CC-CA complex was exposed. The tissue complex was removed and transferred into 1 ml ice cold methanol taken in an eppendorf tube. About 50 such tissue complexes were collected in an eppendorf tube. The tissue complexes were homogenized by ultrasonication for a period of 4 min at 0 °C. The tissue homogenate was then centrifuged at a speed of 10,000 rpm for 5 min. After centrifugation, the supernatant was transferred to another eppendorf tube. The residue was re-extracted with 500 µl of methanol and again centrifuged. The supernatants were combined and solvent from the combined supernatants was removed by keeping in a desiccator. The residue was then taken up in 25 µl of 80% methanol (referred to as CC extract) for RP-HPLC fractionation.

3.2.4. Preparation of crude extract of brain-CC-CA complex of *I. limbata* for bioassays

The brain-CC-CA complex of *I. limbata* was removed as detailed above. Several such tissue complexes were collected into distilled water taken in an eppendorf (5 µl per complex). The tissues were then minced using a fine clean needle and tweezers. The mixture was kept in a steam bath for 4 min in order to

completely extract adipokinetic peptides in the tissue complex and also for denaturing any hydrolytic enzyme that might be present. After bringing the extract to room temperature, tissue fragments were separated by centrifugation at 8000 rpm for 6 min. The supernatant was removed into another eppendorf tube (now referred to as CC extract) and was used as the source of endogenous hyperlipaemic factor. Different concentrations of CC extract were prepared using double distilled water and kept frozen until use. The CC extract concentration is expressed as gland pair equivalent (gpe).

3.2.5. RP-HPLC fractionation of crude extracts of brain-CC-CA complex of *I. limbata*

Extract made from 40 brain-CC-CA complexes of adult *I. limbata* was fractionated by RP-HPLC, on a Shimadzu HPLC system. The HPLC system was equipped with a Phenomenex Luna reverse phase C_{18} column (length, 250 mm; i.d., 4.6 mm; particle size, 5 µm; pore diameter, 100 Å) which was guarded by a 4 mm × 3 mm (i.d.) guard column containing C_{18} material (5 µm). The binary system having 2 pumps (LC.10AT vp) was operated by the software, Shimadzu Class vp.

A sample (20 µl) of the crude extract of brain-CC-CA complexes (containing approximately 2 gpe/µl) was injected into the HPLC system and the programme was operated in a binary gradient of 43% to 53% of solvent B (60% acetonitrile in 0.05% TFA) against solvent A (0.05% aqueous TFA) over 20 min with a flow rate of 1.0 ml/min. The peaks were detected with a UV

detector (SPD-10A vp/10AV vp) at 210 nm. One minute fractions were collected manually in 20 separate eppendorf tubes.

3.2.6. *In vivo* bioassays

For *in vivo* assays, haemolymph samples were collected from the cut end of the antenna using drawn out and calibrated Pasteur pipettes. Before treatment, haemolymph sample (2-4 µl) was collected from one antenna and transferred into the bottom of a 5 ml test tube for estimating the basal level of haemolymph lipids (control). Immediately after the collection of the initial haemolymph sample, test solution was injected into the insect through the lateral pleural membrane between the second and third abdominal segments using a Hamilton microsyringe. The needle was kept in the puncture for a while before withdrawal of the syringe, to allow the material to mix with the haemolymph and also to avoid any loss of test material and haemolymph through the puncture. A second haemolymph sample (experimental) of 2-4 µl was collected from the cut end of the other antenna, 35 min after injection, for lipid estimation.

3.2.7. Preparation of fat body for *in vitro* incubation

For *in vitro* experiments, fat body for the experimental as well as the control was taken from the same insect to minimize individual variation in fat body response. Using surgical scissors, insect was cut laterally and the whole tergum was removed. Digestive and reproductive organs were removed and

discarded. Fat body was taken out, tracheae were removed from the fat body as much as possible, washed in calcium free incubation buffer and blotted on a piece of blotting paper. Keeping on a glass disc, the fat body was chopped with a sharp razor blade into small pieces. The chopped fat body pieces were mixed thoroughly and divided into two approximately equal halves, one half served as the experimental and the other as control.

3.2.8. *In vitro* incubation of fat body

Flat bottomed glass vials (5 ml capacity) with bakelite screw caps were used for *in vitro* incubation of fat body. Each half of the chopped fat body was transferred into separate preweighed incubation vials containing fixed volumes (200 µl) of HEPES buffer (with or without $CaCl_2$) with the necessary additions. The weight of the fat body was then determined. The incubation was carried out for 35 min in a shaker water bath, set at 37 °C. In controls, equivalent volumes of solvents of the corresponding test material added in the experimental, were included in the incubation medium to enable comparison.

3.2.9. Extraction of lipids released from the fat body

After *in vitro* incubations, lipids released into the incubation medium were extracted by a modified Bligh and Dyer (1959) procedure as detailed below. Using a micropipette, 150 µl of the incubation medium was drawn from the incubation vial without trapping any fat body pieces. The drawn out sample was transferred into a centrifuge tube and deproteinised with 500 µl of

chloroform-methanol mixture and was allowed to stand for at least 15 min. To the deproteinised sample, 500 µl of sodium chloride (1 M) followed by 500 µl of chloroform were added. The mixture was shaken well and centrifuged for 3 min at 1000 rpm to separate the organic and aqueous phases. The lower organic phase contained the extracted lipids. A sample (100 µl) was drawn from the lower organic phase using micropipette and transferred into a test tube for quantitation of lipids.

3.2.10. Quantitation of extracted lipids from *in vitro* incubation

From the lipid sample mentioned above, chloroform was evaporated off by keeping it at 48 °C in a hot air oven. The amount of lipids in the samples was measured by the sulphophosphovanillin reaction as total phosphovanillin positive materials (Frings *et al*., 1972). After evaporation, the lipid residues left were mixed with conc. H_2SO_4 (100 µl) and heated in a boiling water bath for 10 min to oxidize lipids into ketones. The test tube was cooled to room temperature and phosphovanillin reagent (5 ml) was added. The ketones develop a pink colour with phosphoric acid and vanillin. The contents were mixed well and incubated at 30 °C for 5 min. The absorbance was read against a reagent blank at 540 nm using spectrophotometer within 10 min.

3.2.11. Quantitation of haemolymph lipids

Total lipids in the haemolymph samples of *in vivo* experiments were determined using the phosphovanillin reagent. Haemolymph samples (2-4 µl)

were directly mixed with 50 µl conc. H_2SO_4 in test tubes and heated in a boiling water bath for 10 min. After cooling to room temperature, 2 ml of phosphovanillin reagent were added to each test tube, thoroughly mixed and were incubated at 30 °C for 5 min. The absorbance at 540 nm were determined within 10 min.

3.2.12. Quantitation of lipid standards

For *in vitro* studies, known quantities of glycerol trioleate were taken in test tubes, added 100 µl of conc. H_2SO_4 to each tube and thoroughly mixed to oxidise the entire glycerol trioleate residue. After keeping in a boiling water bath for 10 min, the mixture was brought to room temperature and phosphovanillin reagent (5 ml) was added, mixed properly and incubated as mentioned above. The absorbance against reagent blank was recorded at 540 nm.

For *in vivo* studies, the same procedure as above was used except that the volume of conc. H_2SO_4 was 50 µl instead of 100 µl and 2 ml phosphovanillin instead of 5 ml.

3.2.13. *In vivo* bioassays to analyse the adipokinetic activity of HPLC fractions

Solvents were removed off from all the 20 fractions collected from HPLC using freeze dryer. The freeze dried materials were resuspended in 200 µl of double distilled water and stored until use. Each of the 20 fractions was

tested for its ability to mobilise lipids from the fat body of *I. limbata* by injecting 10 µl dose (2 gpe/10 µl) each into six assay insects. Collection of haemolymph samples before (control) and 35 min after injection of the test material (experimental) were done as described in section 3.2.6.

3.2.14. *In vivo* bioassay to study the effect of Ca^{2+} on lipid release from the fat body

To examine the lipid releasing effect of Ca^{2+} on the fat body of *I. limbata*, 15 µl each of the different concentrations of $CaCl_2$ was injected into the haemocoel of different insects, considering that the average total haemolymph volume of *I. limbata* (as estimated) is approximately 60 µl to get final concentrations of 1.0, 1.5, 2.0, 4.0 or 8.0 mM $CaCl_2$. Haemolymph samples were collected before and 35 min after injection of $CaCl_2$, for lipid quantitation.

3.2.15. *In vitro* bioassay to study the effect of Ca^{2+} on lipid release from the fat body

For studying the effects of Ca^{2+} on mobilization and release of lipids from the fat body of *I. limbata in vitro*, one half each of the fat bodies were incubated in 200 µl HEPES buffer mixed with 50 µl of various concentrations of $CaCl_2$ solutions (see section 3.2.2.2) for experimental treatment. For controls, the other halves of the fat bodies from the corresponding insects were incubated in HEPES buffer (200 µl) where calcium was omitted but the volume

of the incubation medium was adjusted by adding distilled water (50 μl). Samples of the incubation medium were taken after 35 min of incubation for the extraction and quantitation of the released lipids.

3.2.16. *In vitro* bioassays to study the modulating effect of Ca^{2+} on Lom-AKH-I action on the fat body

In order to study the Ca^{2+} dependence of Lom-AKH-I-mediated lipid release, fat bodies were incubated in HEPES buffer without $CaCl_2$, but with various concentrations of Lom-AKH-I (50, 100 or 200 pmol/10 μl) for experimental. In controls, 10 μl of distilled water was added instead of Lom-AKH-I. After 35 min, incubation media were collected for quantitation of released lipids.

To examine the dose response of $Ca^{2+}{}_o$ on modulating the action of Lom-AKH-I, fat bodies were incubated for 35 min in incubation buffers (200 μl) containing Lom-AKH-I (10 μl) and various concentrations of $CaCl_2$ (50 μl) (section 3.2.2.2). A concentration of 100 pmol/10 μl Lom-AKH-I was selected for this bioassay, based on the dose response results in the *in vitro* bioassay for paired and matched fat body pieces of *I. limbata* (Rasheed and Gokuldas, 2002). A control was maintained for each set of experiment where Lom-AKH-I was replaced by same volume of distilled water. After incubation, known volumes of the medium were collected for extraction and quantitation of released lipids.

In the third set of experiments, designed to study the combined effect of Ca^{2+} and Lom-AKH-I on lipid mobilization, the fat bodies were incubated in the medium containing Lom-AKH-I (100 pmol/10 μl) and various concentrations of $CaCl_2$ (in 50 μl) whereas in the controls, hormone and $CaCl_2$ were replaced by 60 μl of distilled water. After incubation, 150 μl each of the incubation medium, from experimental and control were drawn for estimation of the released lipids.

3.2.17. *In vitro* bioassays to study the modulating effect of Ca^{2+} on the action of native CC extract on the fat body

To determine the effect of native CC extract on lipid mobilization and release in the absence of Ca^{2+}_o, one half of the chopped fat body was incubated in 200 μl of the Ca^{2+}-free HEPES buffer and 10 μl of various concentrations of CC extract (0.5, 1.0 and 2.0 gpe) which served as the experimental. The other half of the fat body was mixed with same quantity of Ca^{2+}-free buffer and 10 μl of distilled water to serve as the control. After incubation at 37 °C for 35 min, the incubation buffer was drawn for estimating the released lipids.

To investigate the effect of Ca^{2+}_o concentration on CC extract-mediated lipid mobilization and release, one half of the fat body was incubated in HEPES buffer containing various Ca^{2+} concentrations (1.0, 1.5, 2.0, 4.0 or 8.0 mM) and CC extract (10 μl) (experimental) and the other half of the fat body from the same insect was incubated in HEPES buffer containing various

concentrations of Ca^{2+} in the absence of CC extract (control). Here 1 gpe of CC extract/incubation was used since the fat body of *I. limbata* showed maximum hyperlipaemic response at a concentration of 1 gpe of CC (Rasheed and Gokuldas, 2002). After incubation, samples of the media were drawn from both experimentals and controls for estimation of released lipids.

3.2.18. Effect of Ca^{2+} ionophore, A23187 on lipid mobilization and release from the fat body

To study the effect of Ca^{2+} influx in lipid mobilization from the fat body of *I. limbata*, the fat bodies were incubated in buffer containing Ca^{2+} (1 mM) and various concentrations of the calcium ionophore, A23187 (1, 5, 10, 15, 20 and 25 µM). A control was maintained for each set of experiment by incubating the other half of the autogenous fat body in a Ca^{2+} (1 mM) containing medium where A23187 was replaced by equal volume (5 µl) of DMSO. Concentration of $CaCl_2$ was maintained at 1 mM, which is the normal concentration of $CaCl_2$ in HEPES buffer, to avoid any undesired pharmacological effect caused due to the excessive influx of $Ca^{2+}{}_o$. Incubations were done for 35 min at 37 °C in total darkness. After incubation the amount of lipid released into the incubation medium was determined.

In a second set of experiment, the effect of A23187 on lipid mobilization and release from the fat body in the absence of Ca^{2+} was studied. Fat bodies were treated with optimum concentration of A23187 (as demonstrated from the

previous experiment) in a Ca^{2+} free medium. The controls contained 5 µl of DMSO instead of ionophore.

In the third set of experiment, the cumulative effect of Ca^{2+}, Lom-AKH-I and A23187 on lipid release from the fat body was studied. Fat body pieces were incubated in a Ca^{2+} (1 mM) containing medium with Lom-AKH-I (100 pmol/10 µl) and calcium ionophore (15 µM; 5 µl). For controls, the same incubation medium with Lom-AKH-I and 5 µl of DMSO was used.

The experiment was repeated, but with Lom-AKH-I replaced by CC extract (1 gpe/incubation). After incubation in darkness, the incubation media were collected for lipid estimation.

3.2.19. Antagonistic effect of La^{3+} on Ca^{2+}-induced lipid release from the fat body

To determine if a disruption in Ca^{2+} influx will modify Ca^{2+} induced lipid release from the fat body of *I. limbata*, various concentrations of La^{3+} (as $LaCl_3$; 0.1, 0.5, 1.0, 1.5 or 2.0 mM) were included (50 µl) in the fat body incubation medium as a plasma membrane Ca^{2+} channel blocker. Concentration of $CaCl_2$ was 1 mM. For controls $LaCl_3$ was replaced by equal volumes of double distilled water. The lipid released into the medium was quantitated in both experimental and control.

3.2.20. Effect of Ca^{2+} channel antagonists on CC extract-induced lipid release from the fat body

To evaluate the effect of inorganic non-specific Ca^{2+} channel antagonists on the levels of CC extract-induced lipid release from the fat body of *I. limbata* in the presence of Ca^{2+}, fat bodies were incubated in HEPES buffer with 1 mM $CaCl_2$, CC extract (1 gpe in 10 µl) and various concentrations (0.1, 0.5, 1.0, 1.5 or 2.0 mM) of Ca^{2+} channel blockers. Lanthanum ions and Cd^{2+} (as $CdCl_2$) were tested as the Ca^{2+} channel blockers in separate bioassays. For controls, the solutions of lanthanum chloride and cadmium chloride were replaced by equal volumes (50 µl) of double distilled water. After *in vitro* incubation of the fat body, lipids released into the incubation medium were quantitated.

3.2.21. Effect of calcium chelator, EGTA on lipid release

Four sets of *in vitro* experiments were designed to analyse the effect of reduction of Ca^{2+}_o level on Ca^{2+}_o-mediated lipid release and also on the modulating effect of Ca^{2+}_o on the action of Lom-AKH-I- and CC extract-mediated lipid release from the fat body. In these experiments, Ca^{2+}_o were chelated with EGTA, a calcium specific chelator, included in the incubation buffer.

In the first set of experiments, to find out the effect of trace amounts of Ca^{2+}, which may be present in the incubation mixture, on lipid release, the fat bodies were incubated in a calcium free HEPES buffer without added EGTA

(experimental). For the control, the medium contained added EGTA (2 mM, 20 µl).

In the second set of experiments, in the experimental tests, fat bodies were incubated in HEPES buffer containing Ca^{2+} (as $CaCl_2$, 1.5 mM, 20 µl), but without EGTA. In control incubations, $CaCl_2$ was replaced by equal volume of 2 mM EGTA.

The third set of experiments was designed to investigate the effect of Lom-AKH-I on lipid release from the fat body of *I. limbata*, when Ca^{2+}_o was chelated using EGTA. Here for the experimental, one half of the fat body was incubated with Lom-AKH-I (100 pmol/10 µl) in the presence of 2 mM EGTA (20 µl). In the control, Lom-AKH-I was replaced by 10 µl of distilled water.

In the last set of experiments, the same procedure as for the third set was used. However, instead of Lom-AKH-I, CC extract (1 gpe, 10 µl) was included in the incubation mixture.

3.2.22. Effect of thimerosal on lipid mobilsation and release from the fat body

To investigate the role of intracellular Ca^{2+} in lipid mobilization and release from the fat body of *I. limbata*, hyperlipaemic response of the fat body to different concentrations of an IP_3R-sensitive thiol reagent, thimerosal was tested. For experimentals, one half of the chopped fat body was incubated in a

calcium free medium containing thimerosal (25, 50, 100, 150 or 200 µM). In controls, thimerosal solution was replaced by double distilled water (20 µl).

3.2.23. Calculations

From the absorbance values obtained from spectrophotometry, concentrations of lipids in various samples were calculated by applying the following formula.

$$\text{Concentration of sample} = \frac{\text{Concentration of standard x Absorbance of sample}}{\text{Absorbance of standard}}$$

From this value, the amount of lipid released into unit volume of haemolymph in the case of *in vivo* experiments and the amount of released lipids by unit weight of fat body into the incubation medium in the case of *in vitro* experiments were calculated. From the values thus obtained from experimentals and controls, the changes in the pattern of lipid release due to the action of various agents were estimated.

3.2.24. Statistical analysis of data

The data obtained from various experiments described include controls and experimentals from which the percentage of difference of the experimentals over the controls (E/C %) brought about by specific agents were calculated. The values were expressed as means ± standard error of means (SEMs). The significance of differences between controls and experimentals

were determined by Student's t-test. Those having P value ≤ 0.05 were considered significant.

Since the data were found to follow normality assumptions, one way analysis of variance (ANOVA) was used to compare the means obtained from different experiments. Here also, P value ≤ 0.05 were considered significant.

Statistical analyses of data were performed using SPSS software. The data were plotted as graphs using the computer programme, MicroCal Origin.

Chapter 4

Results

RESULTS

1. Effect of Ca^{2+}_o on lipid release from the fat body

Experiments were conducted to assess the influence of Ca^{2+}_o on basal level of lipid mobilization and release from the fat body of *I. limbata in vitro*. The quantitative change in lipids released into the incubation medium under the influence of five concentrations of calcium (as calcium chloride), ranging from 1 to 8 mM, were measured and the results are provided in Table IV-1. The results show that Ca^{2+}_o alone, in the absence of any added lipid mobilizing agonists, can influence the release of lipids from the fat body. In *I. limbata*, this effect of calcium is dose dependent (Figure IV-1). It was observed that, lower concentrations of calcium chloride (1.0, 1.5 and 2.0 mM) stimulated the fat body to mobilize and release lipids. A concentration of 1.5 mM of calcium chloride gave the maximum increase in lipid release (28%) which was statistically significant ($P<0.05$). Significant increase in lipid release was also noticed with other calcium chloride concentrations such as 1 and 2 mM. Increase in lipid release was about 25% with 2 mM which was highly significant ($P<0.005$). With 1 mM calcium chloride also the effect was significant ($P<0.05$), though the stimulation of lipid release was lower (18%). With high concentrations, calcium failed to activate the fat body to release lipids. When fat body was incubated in a medium containing 4 mM calcium, lipid release was significantly reduced ($P<0.01$) to 87% of the control sample.

The highest concentration of calcium tested (8 mM) also had inhibitory effect on lipid release (E/C = 97%), but was not statistically significant.

The data obtained for the *in vitro* experiments were subjected to one way ANOVA and the results are provided in Table IV-2. The analysis of the data shows that difference between the effects of different concentrations of Ca^{2+} on lipid release is highly significant ($P<0.000$).

To further substantiate whether the effects of Ca^{2+}_{o} on lipid release from the fat body can be reproduced *in vivo* conditions, various concentrations of calcium chloride were injected into *I. limbata* and the differences in lipid release were measured. The data are presented in Table IV-3 and the dose response curve is presented in Figure IV-2. The values obtained from *in vivo* experiments were found to be similar to those obtained from *in vitro* experiments. Injection of 1 mM calcium chloride into *I. limbata* increased haemolymph lipid level significantly ($P<0.05$) by 18%. A slight but significant ($P<0.005$) increase (20%) in stimulation was noticed when the concentration of calcium chloride was increased to 1.5 mM. Maximum response to calcium (28%) was obtained with a concentration of 2 mM ($P<0.05$). Similar to that of *in vitro* conditions, *in vivo* treatment with higher concentrations of calcium chloride (4 and 8 mM) also showed reduction in lipid release. For example, with a calcium chloride concentration of 8 mM, the lipid release was reduced to 85% of control values ($P<0.05$).

The means of the variations in the amount of lipid released under the influence of different concentrations of calcium were compared by one way ANOVA and the details are shown in Table IV-4. The data suggest that the variation in the amount of lipid release due to change in calcium concentration *in vivo* is significant.

2. Effect of Ca^{2+}_{o} on Lom-AKH-I-mediated lipid release from the fat body

The effects of various concentrations of Lom-AKH-I on lipid release from the fat body of *I. limbata in vitro* were tested in an incubation medium devoid of calcium chloride. The results obtained are tabulated in Table IV-5 and the pattern of change in lipid release is represented in Figure IV-3. The *in vitro* studies show that Lom-AKH-I has only a negligible effect on lipid release from the fat body of *I. limbata* in the absence of Ca^{2+}_{o}, and all the three concentrations of Lom-AKH-I (50, 100 and 200 pmol) failed to induce any appreciable level of lipid release from the fat body. The analysis of the data showed that differences between the experimental and control tests in the case of 100 and 200 pmol of Lom-AKH-I were statistically not significant. However, the stimulation of lipid release attained with 50 pmol, though very small, appeared to be statistically significant ($P<0.05$).

From the above experiment it appears that the hyperlipaemic action of Lom-AKH-I in *I. limbata* is dependent on the presence of Ca^{2+}_{o}. So in the subsequent experiments, effects of Lom-AKH-I in the presence of various concentrations of calcium chloride were assessed. The effects of increasing

concentrations of calcium on Lom-AKH-I-mediated lipid release from the fat body of *I. limbata* were compared to the values obtained with different concentrations of calcium, in the absence of hormone (Table IV-6). The effect on the pattern of lipid release is shown graphically in Figure IV-4. The results suggest that the adipokinetic activity of Lom-AKH-I (100 pmol) on the fat body of *I. limbata* is dependent on the dose of Ca^{2+}_{o}. With lower concentrations of calcium, Lom-AKH-I exhibited distinct stimulatory effects on the lipid release. With 1 mM calcium, Lom-AKH-I had a moderately high stimulatory effect even though it was not statistically significant. Significantly high stimulation was observed at concentrations of 1.5 mM ($P<0.05$) and 2.0 mM ($P<0.01$). The maximum activity was obtained at a calcium chloride concentration of 1.5 mM where Lom-AKH-I increased lipid release by 34% as that of the control, which contained 1.5 mM calcium, and no Lom-AKH-I. At high concentrations of calcium (4 and 8 mM), Lom-AKH-I seemed to have no adipokinetic activity on the fat body of *I. limbata*.

The mean values obtained were subjected to ANOVA and the details are presented in Table IV-7. The data reveal that the change in the lipid release caused by a change in the concentration of calcium is highly significant ($P<0.001$).

In another set of experiments, the lipid released from the fat body of *I. limbata* in response to different concentrations of Ca^{2+}_{o} and Lom-AKH-I was compared with similar values obtained from control incubations where both

calcium and hormone were absent (Table IV-8). It was observed that lower concentrations of calcium together with Lom-AKH-I elicited a cumulative effect on lipid release. The hormone elicited a significant (P<0.0005) rise of 39% in lipid release, when the fat body of *I. limbata* was incubated in a medium containing 1 mM calcium. This value was found to be higher than the sum of the values obtained from the individual effects of 1 mM calcium chloride (Table IV-1) and 100 pmol Lom-AKH-I (in the presence of 1 mM $CaCl_2$) (Table IV-6). Inclusion of 1.5 mM calcium enhanced Lom-AKH-I induced lipid release by 57%, which is only slightly less than the total of the individual effects of 1.5 mM calcium (28%) (Table IV-1) and of 100 pmol of Lom-AKH-I in the presence of 1.5 mM calcium (34%) (Table IV-6). Similarly, 2 mM calcium also showed a significant (P<0.005) added effect, when combined with Lom-AKH-I. Further increase in calcium concentrations seemed to have blocked the stimulatory effect of Lom-AKH-I on lipid release (Figure-IV-5).

One way ANOVA of net change in lipid release owing to Lom-AKH-I for each concentration of calcium (Table IV-9) showed that the variation in lipid release is significantly different (P<0.000).

3. RP-HPLC fractionation of crude extract of brain-CC-CA complex of *I. limbata*

The methanolic extract of forty brain-CC-CA complexes of *I. limbata* was subjected to RP-HPLC fractionation as described in section 3.2.5 and the

elution profile of the chromatography was recorded (see Figure IV-6). Elution profile clearly shows a number of small and large UV absorbing peaks. Out of these, four peaks were identified (numbered 1-4) as major peaks with retention times of 6.34, 8.29, 9.39 and 10.35 min respectively. Chromatogram shows the presence of many peptides in the brain-CC-CA complex of *I. limbata*. Comparing the peaks and the peak areas, it becomes apparent that the peaks with the retention times of 9.39 and 10.35 min have more materials than other peaks.

4. Analysis of HPLC fractions of CC extract of *I. limbata* for their adipokinetic activity

The results of the *in vivo* bioassays with twenty HPLC fractions of CC extract of *I. limbata* are shown in Table IV-10 and the extent of adipokinetic activities of these fractions are presented in Figure IV-7. Results showed that fractions 7, 8, 11 and 12 have significant hyperlipaemic activity. Highest hyperlipaemic activity was shown by the fraction 7, which induced the fat body to release 32% ($P<0.01$) more lipids than the controls. Fraction 8 elicited 23% increase in lipid release when injected in to the experimental insect ($P<0.01$). Though the fraction 9 was responsible for an increase in release of lipids by 20%, it was not found to be statistically significant. Both the fractions 11 and 12, elicited 16% increase in lipid release into the haemolymph, which was statistically significant ($P<0.05$). Fraction 18 was found to be slightly hypolipaemic. However, the effect was not significant. None of the other

fractions showed any significant adipokinetic activity. Thus, the *in vivo* bioassays with HPLC fractions showed that the brain-CC-CA complex of *I. limbata* contains adipokinetic factors. Results also indicated that more than one factor of the extract had adipokinetic activity.

5. The effect of Ca^{2+}_{o} on CC extract-mediated lipid release from the fat body

The influence of Ca^{2+}_{o} on CC extract-mediated lipid release from the fat body of *I. limbata* was examined with two sets of experiments. In the first set, the effect of CC extract was studied in the absence of calcium while the second set was conducted in the presence of calcium.

The data obtained from the *in vitro* experiments to evaluate the effects of various concentrations of CC extract of *I. limbata* on lipid release in the absence of Ca^{2+} in the incubation medium is recorded in the Table IV-11 and is graphically shown in Figure IV-8. The data clearly show that in the absence of Ca^{2+}_{o}, CC extract (0.5, 1.0 and 2.0 gpe) could not bring in any significant effect on lipid release from the fat body.

In the second set of *in vitro* experiments, the influence of Ca^{2+}_{o} on the CC extract-mediated adipokinetic activity, and the extent to which calcium modify this lipid release from the fat body of *I. limbata* were investigated as detailed in section 3.2.17. The data obtained (Table IV-12 and Figure IV-9) show clearly that Ca^{2+}_{o} modulated the CC extract-mediated *in vitro* lipid

release and the effect of calcium was dose dependent. There was an increase in lipid release when the fat body was incubated with lower concentrations of calcium chloride in the presence of CC extract. The stimulation of lipid release by 1.0, 1.5 and 2.0 mM calcium chloride were more or less of the same magnitude i.e., 37, 44 and 41% respectively. With higher concentrations of Ca^{2+}_{o} (4 and 8 mM), the stimulatory effect of CC extract was found to be abolished.

ANOVA of the above data is represented in Table IV-13. The results revealed that the difference in the amount of lipid released in the presence of various concentrations of calcium is significantly different.

6. Effect of the calcium chelator, EGTA on lipid release from the fat body

Lipid release from the fat body of *I. limbata* in the absence of calcium with and without added EGTA (2 mM) in the incubation medium were monitored. Results are provided in Table IV-14. There was no significant difference in the lipid release between the two sets of fat body incubation during 35 min of incubation. The basal level lipid release was found unaltered when EGTA was added to the incubation medium. The results indicate that the inclusion of EGTA in the incubation medium and omission of Ca^{2+} from the incubation medium have similar effect on the basal level of lipid release from the fat body of *I. limbata*.

In another set of experiments, the effect of calcium in the incubation medium on lipid release was compared with that in a medium without calcium but with added EGTA. Fat body incubated with 1.5 mM calcium released 33% more lipids than the incubations where calcium was chelated with EGTA (Table IV-14). This value is similar to the values obtained where the effect of calcium on lipid release was studied (Table IV-1). The results thus indicate that the effect of chelation of calcium by EGTA on lipid release is similar to the effect obtained when calcium was omitted from the incubation medium.

Similarly, in the case of Lom-AKH-I and CC extract actions, EGTA blocked the induction of lipid release. The results obtained thus emphasise that Lom-AKH-I- and CC extract-induced lipid release also require Ca^{2+}_{o} (Table IV-14).

7. Effect of calcium ionophore, A23187 on lipid release from the fat body

The effect of a calcium ionophore, A23187 on lipid release from the fat body of *I. limbata* was studied to understand how this lipid release is modified by calcium influx. The difference in the effect of different concentrations of A23187 on lipid release in presence of 1 mM calcium was studied and the results are shown in Table IV-15. Dose response curve for the effects of A23187 is shown in Figure IV-10. As shown in the table, the incubation of fat body with A23187 in the presence of calcium elevated lipid release from the fat body in a concentration dependent manner. Low level of calcium ionophore (1.0 μM) had no significant stimulatory effect on the fat body, but significant

($P<0.05$) lipid release occurred from 5 µM concentration onwards. Increase of 41% and 50% in lipid release were observed with 5 and 10 µM A23187 respectively. Maximal stimulation (56%) was achieved with an ionophore concentration of 15 µM ($P<0.001$). Incubation with 20 and 25 µM ionophore resulted in stimulation of almost an equal extent (50 and 52% respectively). These results indicate that calcium influx alone is sufficient to stimulate lipid release from the fat body in *I. limbata*.

The data obtained on dose effect of A23187 on lipid release were subjected to one-way ANOVA and the results are presented in Table IV-16. The results indicate that significant variation ($P<0.05$) was caused by different doses of A23187 on lipid release from the fat body of *I. limbata*.

The specific requirement of Ca^{2+}_{o} for A23187-mediated lipid release from the fat body of *I. limbata* was examined using 15 µM solution of A23187 as described in section 3.2.18. Results presented in Table IV-17 show that whereas 15 µM A23187 induced a marked lipid release (E/C% 155.64 ± 12.96) in the presence of 1 mM calcium, A23187 failed to induce lipid release in the absence of calcium. The results suggest that the stimulatory activity of A23187 on lipid release is totally dependent on Ca^{2+}_{o} and that the effect is exerted through the modulation of calcium influx.

The result of the combined effect of Lom-AKH-I and A23187 in Ca^{2+} containing incubation medium on lipid release from the fat body is presented in Table IV-17. The data show that a combination of Lom-AKH-I, A23187 and

Ca^{2+} was responsible for a significant ($P<0.05$) increase in lipid release (31%) than the control groups without A23187. The results show that A23187 has a better stimulatory effect on the fat body than the Lom-AKH-I with regard to lipid release and the combination (Lom-AKH-I, A23187 and Ca^{2+}) does not have a cumulative effect.

The combined effect of CC extract and A23187 in the presence of calcium was also studied (see section 3.2.18). The results (Table IV-17) show that this combination (CC extract, A23187 and Ca^{2+}) was responsible for an increase in lipid release from the fat body by only 18% more than the values obtained with CC extract and Ca^{2+} in the absence of A23187. But this value is not statistically significant. The results suggest that a combination of CC extract, A23187 and Ca^{2+} does not cause a cumulative effect on stimulation of fat body to release lipids.

8. Effect of lanthanum as a non specific calcium channel blocker in Ca^{2+} induced lipid release from the fat body

The effect of La^{3+} on $Ca^{2+}{}_{o}$-induced lipid release from the fat body of *I. limbata* was studied. The results are provided in Table IV-18. The dose response curve (Figure IV-11) shows that La^{3+} inhibits Ca^{2+}-induced lipid release in a dose dependent manner. Low concentration of lanthanum (0.1 mM) caused only a very small inhibition of lipid release which was not significant. However, significant inhibition was brought about by 0.5 mM ($P<0.05$) and 1.0 mM ($P<0.005$) lanthanum chloride. Maximum inhibition of lipid release was

exerted by 1.5 and 2.0 mM lanthanum, where the lipid release was reduced to about 78% of the control.

Significance of differences between means was checked by one way ANOVA and the details are shown in Table IV-19. The data shows that inhibition caused due to the change in La^{3+} concentration is significant at $P<0.05$.

9. The effects of nonspecific calcium channel antagonists on CC extract-induced lipid release from the fat body

To further demonstrate the importance of Ca^{2+}_{o} and its influx into the fat body cells on CC extract-induced lipid release from the fat bodies of *I. limbata*, the effect of lanthanum on lipid release from the fat body of the insect was studied as described in section 3.2.20. The results are given in Table IV-20 and the dose response curve is presented in Figure IV-12. It was observed that, lanthanum greatly reduced the CC extract-induced lipid release in a dose dependent manner in *I. limbata*. Lower concentrations of lanthanum (0.1 and 0.5 mM) could not evoke any significant antagonistic effect on CC extract-induced lipid release. Significant ($P<0.05$) inhibition was caused by lanthanum concentration of 1 mM and above. Incubation with 1 mM lanthanum reduced the lipid release to 82% of the control, while 1.5 mM reduced it to 77% of the control. In the presence of 2 mM lanthanum, the lipid release was decreased to 63% ($P<0.0005$).

Analysis of the data by one way ANOVA (see Table IV-21) indicates that the difference in the effects caused by different lanthanum concentrations is significant ($P<0.005$).

The significance of Ca^{2+}_{o} influx was further examined using another non-specific calcium channel blocker, cadmium. The results of the influence of different concentrations of cadmium, on CC extract-mediated lipid release are presented in Table IV-22. Figure IV-13 graphically represents the effects of various concentrations of cadmium. It was seen that all the concentrations of cadmium tried, had inhibitory effects on the lipid release, but significant effect was noticed from 1 mM onwards only. When an equimolar concentration of cadmium (1 mM) as that of Ca^{2+} was included in the incubation medium, the lipid release was reduced to 72.82% of the controls ($P<0.005$). Similar inhibition (70.75%) was caused by 1.5 mM cadmium ($P<0.05$). The maximum inhibition (to 65%) was noticed with 2 mM cadmium, which is statistically highly significant ($P<0.000$). The results indicate that Cd^{2+} is as effective as La^{3+} in inhibiting CC extract-induced lipid release from the fat body and lower concentrations of cadmium (0.1, 0.5, 1.0 and 1.5 mM) gave greater inhibition than the corresponding concentrations of lanthanum.

The data were subjected to ANOVA and the details are shown in Table IV-23. The table shows that the difference in inhibition by different concentrations of cadmium is significant ($P<0.05$).

10. The effect of thimerosal on lipid release from the fat body

To evaluate the importance of Ca^{2+}_i in hyperlipaemic signal transduction, the effect of thimerosal, internal calcium mobiliser, on lipid release was tested in a medium without calcium and the results are presented in Table IV-24. As shown in Figure IV-14, thimerosal exhibits a dose sensitive stimulation of lipid release from the fat body of *I. limbata*. Thimerosal concentration of 25 µM could not evoke a significant stimulation, but 50 µM was sufficient to increase the lipid release by 26%, which was significant ($P<0.05$). The most effective concentration was 100 µM, which resulted in 40% increase in lipid release ($P<0.01$). Increase in the thimerosal concentration to 150 and 200 µM resulted in a gradual decline in the degree of stimulation of lipid release to 30 ($P<0.05$) and 27% ($P<0.005$). It was observed that thimerosal-induced stimulation was independent of Ca^{2+}_o. The results indicate that release of Ca^{2+} from intracellular Ca^{2+} stores triggers lipid mobilization and release from the fat body of *I. limbata*.

The mean values obtained for lipid release with 5 different concentrations of thimerosal as mentioned above were subjected to ANOVA, to test the level of significance. The data of the analysis shown in Table IV-25 indicate that the change in lipid release caused by different concentrations of thimerosal is not statistically significant.

Table IV- 1
Lipid release from the fat body of *I. limbata* at various concentrations of $CaCl_2$ *in vitro*

$CaCl_2$ concentration (mM)	Amount of lipid released (mg/g fat body)		E/C (%)	Significance*
	Experimental (E)	Control (C)		
1.0	291.58 ± 24.35	253.54 ± 24.68	117.71 ± 5.43	P<0.05
1.5	194.29 ± 19.15	156.20 ± 17.98	128.42 ± 8.46	P<0.05
2.0	235.58 ± 13.50	192.66 ± 14.53	124.58 ± 6.22	P<0.005
4.0	237.69 ± 17.99	271.74 ± 15.07	87.41 ± 3.75	P<0.01
8.0	297.01 ± 23.99	305.08 ± 14.49	96.57 ± 5.37	N.S

Values are expressed as means ± SEMs (n=9).
For experimental the fat body was incubated in HEPES buffer with added $CaCl_2$, while for controls $CaCl_2$ was omitted.
*Significance of difference between the controls and experimentals.

Table IV- 3
Lipid release from the fat body of *I. limbata* at various concentrations of $CaCl_2$ *in vivo*

$CaCl_2$ concentration (mM)	Amount of lipid released (µg/µl haemolymph)		E/C (%)	Significance*
	Experimental (E)	Control (C)		
1.0	7.84 ± 1.32	6.46 ± 0.89	117.88 ± 6.08	P<0.05
1.5	4.52 ± 0.27	3.81 ± 0.22	119.78 ± 6.02	P<0.005
2.0	11.28 ± 0.96	9.04 ± 0.68	127.73 ± 10.1	P<0.05
4.0	6.43 ± 0.89	7.32 ± 1.03	90.63 ± 5.34	N.S
8.0	6.25 ± 0.91	7.45 ± 1.01	85.12 ± 4.95	P<0.05

Values are expressed as means ± SEMs (n=12).
Haemolymph samples were collected for lipid estimation before and 35 min after injection of $CaCl_2$, for control and experimental respectively.
*Significance of difference between the controls and experimentals.

Table IV- 6
Lipid release from the fat body of *I. limbata* incubated with Lom-AKH-I and various concentrations of $CaCl_2$ *in vitro*

$CaCl_2$ concentration (mM)	Amount of lipid released (mg/g fat body)		E/C (%)	Significance*
	Experimental (E)	Control (C)		
1.0	212.57 ± 28.94	196.45 ± 33.52	113.07 ± 5.90	N.S
1.5	183.08 ± 17.97	139.50 ± 8.54	134.32 ± 13.63	P<0.05
2.0	249.81 ± 15.26	210.83 ± 15.17	120.45 ± 6.78	P<0.01
4.0	214.88 ± 9.80	224.76 ± 9.85	96.80 ± 5.20	N.S
8.0	182.15 ± 11.55	201.13 ± 16.38	92.28 ± 4.45	N.S

Values are expressed as means ± SEMs (n=9).
For experimental, fat body was incubated in HEPES buffer containing calcium and 100 pmol of Lom-AKH-I; controls lacked Lom-AKH-I.
*Significance of difference between the controls and experimentals.

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Table IV-8
Combined effect of Lom-AKH-I and calcium on lipid release from the fat body of *I. limbata*

$CaCl_2$ concentration (mM)	Amount of lipid released (mg/g fat body)		E/C (%)	Significance*
	Experimental (E)	Control (C)		
1.0	298.38 ± 27.67	214.60 ± 17.66	138.95 ± 4.58	P<0.0005
1.5	220.36 ± 17.29	141.88 ± 9.24	156.63 ± 9.48	P<0.001
2.0	202.75 ± 18.93	145.48 ± 17.30	146.39 ± 11.88	P<0.005
4.0	317.97 ± 19.63	358.89 ± 23.91	89.48 ± 4.42	N.S
8.0	160.13 ± 5.77	177.58 ± 9.58	92.23 ± 6.31	N.S

Vales are expressed as means ± SEMs (n = 9).
Fat bodies were incubated in HEPES buffer containing 100 pmol of Lom-AKH-I and indicated concentrations of $CaCl_2$; controls lacked $CaCl_2$ and Lom-AKH-I.
*Significance of difference between the controls and experimentals.

Table IV-5
Effect of various concentrations of Lom-AKH-I on lipid release from the fat body of *I. limbata* in a Ca^{2+} free medium

Lom-AKH-I concentration (pmol)	Amount of lipid released (mg/g fat body)		E/C (%)	Significance*
	Experimental (E)	Control (C)		
50	187.54 ± 8.35	181.88 ± 8.36	103.21 ± 1.20	P<0.05
100	202.06 ± 28.63	198.74 ± 28.20	101.30 ± 2.89	N.S
200	188.09 ± 17.14	182.21 ± 16.04	103.21 ± 1.82	N.S

Values are expressed as means ± SEMs (n = 7).
For experimental, the fat body was incubated in Ca^{2+} free HEPES buffer containing Lom-AKH-I; controls are devoid of both Ca^{2+} and Lom-AKH-I.
*Significance of difference between the controls and experimentals.

Table IV-12
Effect of native CC extract on lipid release from the fat body of *I. limbata* in the presence of various concentrations of $CaCl_2$

$CaCl_2$ concentration (mM)	Amount of lipid released (mg/g fat body)		E/C (%)	Significance*
	Experimental (E)	Control (C)		
1.0	194.77 ± 21.79	149.02 ± 17.13	137.07 ± 11.72	P<0.05
1.5	200.64 ± 36.87	145.46 ± 26.37	143.66 ± 10.47	P<0.01
2.0	201.65 ± 31.02	148.85 ± 23.56	140.78 ± 10.20	P<0.01
4.0	178.68 ± 18.76	185.60 ± 22.92	93.47 ± 6.32	N.S
8.0	200.74 ± 22.06	201.00 ± 16.70	99.54 ± 7.62	N.S

Values are expressed as means ± SEMs (n=9).
For experimental, fat body pieces were incubated in HEPES buffer containing indicated concentrations of calcium and 1 gpe of CC extract; controls lacked CC extract.
*Significance of difference between the controls and experimentals

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Table IV- 11
Effects of various concentrations of native CC extract on lipid release from the fat body of *I. limbata* incubated in Ca^{2+} free medium

CC extract concentration (gpe)	Amount of lipid released (mg/g fat body)		E/C (%)	Significance*
	Experimental (E)	Control (C)		
0.5	186.33 ± 26.14	191.01 ±26.05	97.58 ± 4.66	N.S
1.0	220.64 ± 11.13	217.02 ± 14.03	102.99 ± 3.93	N.S
2.0	194.40 ± 24.04	183.78 ± 18.42	104.16 ± 4.15	N.S

Values are expressed as means ± SEMs (n=7).
For experimental, fat body was incubated in a Ca^{2+} free medium containing indicated concentrations of CC extract; controls are devoid of both Ca^{2+} and CC extract.
*Significance of difference between the controls and experimentals.

Table IV-18
Effects of nonspecific calcium channel antagonist, lanthanum, on Ca^{2+}-induced lipid release from the fat body of *I. limbata*

$LaCl_3$ concentration (mM)	Amount of lipid released (mg/g fat body)		E/C (%)	Significance*
	Experimental (E)	Control (C)		
0.1	202.43 ± 19.87	228.50 ± 38.53	98.63 ± 10.82	N.S
0.5	195.51 ± 17.58	215.33 ± 21.75	92.24 ± 2.98	P<0.05
1.0	175.60 ± 16.24	214.57 ± 20.63	82.04 ± 3.16	P<0.005
1.5	149.97 ± 14.94	194.74 ± 16.79	77.80 ± 4.69	P<0.005
2.0	136.19 ± 13.00	187.06 ± 25.26	77.19 ± 4.98	P<0.01

Values are expressed as means ± SEMs (n=8).
Fat body was incubated in calcium (1 mM) containing medium with (experimental) or without (control) $LaCl_3$.
*Significance of difference between the controls and experimentals.

Table IV-20
Effect of lanthanum on native CC extract-mediated lipid release from the fat body of *I. limbata* in presence of extracellular Ca^{2+}

$LaCl_3$ concentration (mM)	Amount of lipid released (mg/g fat body)		E/C (%)	Significance*
	Experimental (E)	Control (C)		
0.1	184.77 ± 30.69	180.66 ± 22.40	103.86 ± 10.19	N.S
0.5	205.88 ± 39.23	227.61 ± 35.84	90.75 ± 6.58	N.S
1.0	170.41 ± 28.21	199.81 ± 21.09	82.37 ± 6.38	P<0.05
1.5	153.72 ± 12.89	205.14 ± 19.00	76.66 ± 5.95	P<0.05
2.0	106.69 ± 7.23	170.26 ± 8.65	63.17 ± 4.46	P<0.0005

Values are expressed as means ± SEMs (n=7).
For experimental, fat body was incubated in Ca^{2+} (1 mM) containing medium in the presence of CC extract (1 gpe) at various concentrations of $LaCl_3$; control incubation was without $LaCl_3$ but with CC extract and Ca^{2+}.
*Significance of difference between the controls and experimentals.

Table IV-15
Dose response of A23187 on lipid release from the fat body of *I. limbata* in presence of Ca^{2+}

A23187 concentration (µM)	Amount of lipid released (mg/g fat body)		E/C (%)	Significance*
	Experimental (E)	Control (C)		
1	124.14 ± 17.65	119.71 ± 22.66	107.62 ± 5.27	N.S
5	146.00 ± 19.14	103.04 ± 9.19	141.38 ± 10.37	P<0.05
10	161.55 ± 21.58	109.65 ± 13.99	149.93 ± 10.55	P<0.01
15	152.51 ± 15.14	99.45 ± 9.24	155.64 ± 12.96	P<0.001
20	161.50 ± 16.82	109.30 ± 11.07	150.38 ± 9.98	P<0.005
25	128.73 ± 11.33	89.55 ± 10.51	151.74 ± 14.45	P<0.005

Values are expressed as means ± SEMs (n=9).
Fat body was incubated in HEPES buffer containing 1 mM $CaCl_2$; experimentals with indicated concentrations of A23187 and controls without A23187.
*Significance of difference between the controls and experimentals.

Table IV-22
Effect of cadmium on native CC extract-mediated lipid release from the fat body of *I. limbata* in the presence of extracellular Ca^{2+}

$CdCl_2$ concentration (mM)	Amount of lipid released (mg/g fat body)		E/C (%)	Significance*
	Experimental (E)	Control (C)		
0.1	187.43 ± 10.96	197.75 ± 14.27	96.89 ± 6.18	N.S
0.5	112.90 ± 13.21	158.09 ± 31.38	77.65 ± 6.86	N.S
1.0	162.11 ± 22.37	220.01 ± 21.70	72.82 ± 6.47	$P<0.005$
1.5	117.14 ± 14.40	177.55 ± 23.79	70.75 ± 8.80	$P<0.05$
2.0	148.93 ± 18.08	224.76 ± 18.80	65.38 ± 3.28	$P<0.000$

Values are expressed as means ± SEMs (n=7).
For experimental, fat body was incubated in Ca^{2+} (1 mM) containing medium in the presence of CC extract (1 gpe) at various concentrations of $CdCl_2$; control incubation was without $CdCl_2$ but with CC extract and Ca^{2+}.
*Significance of difference between the controls and experimentals.

Table IV-24
Effect of various concentrations of thimerosal on lipid release from the fat body of *I. limbata*

Thimerosal concentration (µM)	Amount of lipid released (mg/g fat body)		E/C (%)	Significance*
	Experimental (E)	Control (C)		
25	158.91 ± 22.51	136.35 ± 19.50	116.59 ± 8.99	N.S
50	211.23 ± 31.54	165.70 ± 24.96	126.28 ± 12.59	P<0.05
100	229.37 ± 30.03	170.64 ± 28.07	140.13 ± 9.93	P<0.01
150	170.78 ± 31.99	130.78 ± 27.28	129.59 ± 9.64	P<0.05
200	220.24 ± 25.50	178.65 ± 23.07	126.71 ± 7.11	P<0.005

Values are expressed as means ± SEMs (n=9).
Fat body was incubated in Ca^{2+}-free HEPES buffer either in the presence of indicated concentrations of thimerosal (experimental) or in the absence of thimerosal (control).
*Significance of difference between the controls and experimentals.

Table IV-10
Analysis of HPLC fractions of CC extract of *I. limbata* for their hyperlipaemic activity

Fractions	Amount of lipid released (µg/µl haemolymph)		E/C (%)	Significance*
	Experimental (E)	Control (C)		
1	4.93 ± 1.15	4.30 ± 0.83	109.65 ± 9.07	N.S
2	5.90 ± 1.39	5.35 ± 1.06	107.55 ± 7.49	N.S
3	4.90 ± 0.74	4.42 ± 0.67	112.48 ± 5.91	N.S
4	5.81 ± 0.79	5.47 ± 0.71	106.56 ± 5.96	N.S
5	6.54 ± 1.37	5.87 ± 1.13	101.15 ± 3.58	N.S
6	3.61 ± 0.24	3.64 ± 0.31	100.30 ± 4.22	N.S
7	3.83 ± 0.30	3.00 ± 0.39	131.51 ± 8.27	P<0.01
8	3.97 ± 0.62	3.25 ± 0.51	122.47 ± 4.11	P<0.01
9	4.87 ± 0.64	4.06 ± 0.46	120.05 ± 9.69	N.S
10	5.08 ± 0.43	4.06 ± 0.46	107.89 ± 8.64	N.S
11	6.14 ± 0.42	5.37 ± 0.47	115.62 ± 4.40	P<0.05
12	4.20 ± 0.34	3.72 ± 0.42	115.66 ± 6.22	P<0.05
13	4.31 ± 0.77	3.97 ± 0.67	108.12 ± 7.99	N.S
14	5.21 ± 0.77	5.27 ± 0.79	97.45 ± 12.90	N.S
15	3.35 ± 0.61	3.20 ± 0.49	102.59 ± 5.33	N.S
16	5.12 ± 0.53	4.79 ± 0.59	108.97 ± 4.78	N.S
17	5.01 ± 0.82	4.50 ± 0.27	109.52 ± 12.54	N.S
18	3.86 ± 0.50	4.45 ± 0.36	86.93 ± 7.91	N.S
19	5.45 ± 0.75	5.10 ± 0.52	107.15 ± 8.82	N.S
20	6.45 ± 0.60	7.09 ± 0.74	93.69 ± 9.43	N.S

Values are expressed as means ± SEMs (n=6).

Haemolymph lipids were measured before and 35 min after injection of 10 µl (2 gpe) of the sample. The samples were prepared by reconstituting each of the freeze dried HPLC fractions of CC extract in 200 µl of distilled water.

*Significance of difference between the controls and experimentals.

Table IV-14
Effects of EGTA as a Ca^{2+} specific chelator on lipid release from the fat body of *I. limbata in vitro*

Treatment	Amount of lipid released (mg/g fat body)	E/C (%)	n	Significance*
-EGTA -Ca^{2+}	186.61 ± 17.19	97.36 ± 3.69	9	N.S
+EGTA -Ca^{2+}	195.06 ± 20.74			
-EGTA +Ca^{2+}	153.04 ± 16.66	133.38 ± 9.73	9	P<0.05
+EGTA -Ca^{2+}	117.09 ± 13.13			
+EGTA +AKH-I	125.46 ± 11.17	97.76 ± 6.51	7	N.S
+EGTA -AKH-I	131.14 ± 13.36			
+EGTA +CC	148.49 ± 30.04	101.24 ± 5.48	9	N.S
+EGTA -CC	142.78 ± 21.91			

Values are expressed as means ± SEMs.
Concentrations of the reagents used: EGTA - 2.0 mM; $CaCl_2$ (Ca^{2+}) - 1.5 mM; Lom-AKH-I - 100 pmol; CC extract - 1.0 gpe.
First line of the each couple of treatment represents the experimental (E) and second line represents control (C).
*Significance of the difference between the controls and experimentals.

Table IV-17
Effect of A23187 on lipid release in the presence and in the absence of different lipid mobilizing agonists

Sl. No.	Treatment	Amount of lipid release (mg/g fat body)	E/C (%)	Significance*
1	+ Ca^{2+}, +A23187 + Ca^{2+}, -A23187	152.51 ± 15.14 99.45 ± 9.24	155.64 ± 12.96	P<0.001
2	- Ca^{2+}, +A23187 - Ca^{2+}, -A23187	147.41 ± 23.13 146.16 ± 21.30	102.39 ± 6.87	N.S.
3	+Ca^{2+}, +AKH-I, +A23187 +Ca^{2+}, +AKH-I, -A23187	200.71 ± 18.23 158.46 ± 16.10	131.31 ± 12.58	P<0.05
4	+ Ca^{2+}, +CC, +A23187 + Ca^{2+}, +CC, -A23187	170.73 ± 28.93 141.35 ± 17.30	117.76 ± 9.36	N.S.

Values are expressed as means ± SEMs (n=9).
Concentrations of the reagents used: $CaCl_2$ (Ca^{2+}), 1 mM; A23187, 15 µM; Lom-AKH-I, 100 pmol; CC extract, 1 gpe.
First line of the each couple of treatment represents the experimental (E) and second line represents control (C).
*Significance of the difference between the controls and experimentals.

Table IV-2
ANOVA of data for *in vitro* lipid release from the fat body of *I. limbata* incubated with various concentrations of $CaCl_2$

Source	Sum of Squares	df	Mean Square	F	Sig. (P)
Between Groups	11819.503	4	2954.876		
Within Groups	13151.367	40	328.784	8.987	0.000
Total	24970.870	44			

Table IV-4
ANOVA of data for *in vivo* lipid release from the fat body of *I. limbata* in presence of various concentrations of $CaCl_2$

Source	Sum of Squares	df	Mean Square	F	Sig. (P)
Between Groups	17409.876	4	4352.469		
Within Groups	30178.918	55	548.708	7.932	0.000
Total	47588.793	59			

Table IV-7
ANOVA of data for *in vitro* lipid release from the fat body of *I. limbata* incubated with Lom-AKH-I at various concentrations of $CaCl_2$

Source	Sum of Squares	df	Mean Square	F	Sig. (P)
Between Groups	11879.134	4	2969.783		
Within Groups	20450.984	40	511.275	5.809	0.001
Total	32330.118	44			

Table IV-9
ANOVA of data for *in vitro* effect of Lom-AHK-I and calcium on lipid release from the fat body of *I. limbata* over a hormone and calcium free medium

Source	Sum of Squares	df	Mean Square	F	Sig. (P)
Between Groups	35893.095	4	8973.274		
Within Groups	22421.583	40	560.540	16.008	0.000
Total	58314.678	44			

Table IV-13
ANOVA of data for *in vitro* lipid release from the fat body of *I. limbata* incubated with native CC extract at various concentrations of $CaCl_2$

Source	Sum of Squares	df	Mean Square	F	Sig. (P)
Between Groups	21293.608	4	5323.402		
Within Groups	32953.805	40	823.845	6.462	0.000
Total	54247.413	44			

Table IV-16
ANOVA of data for *in vitro* lipid release from the fat body of *I. limbata* incubated with various concentrations of A23187 in the presence of calcium

Source	Sum of Squares	df	Mean Square	F	Sig. (P)
Between Groups	14336.138	5	2867.227		
Within Groups	52066.683	48	1084.723	2.643	0.034
Total	66402.819	53			

Table IV-19
ANOVA of data for *in vitro* lipid release from the fat body of *I. limbata* incubated with various concentrations of $LaCl_3$ in the presence of calcium

Source	Sum of Squares	df	Mean Square	F	Sig. (P)
Between Groups	2864.786	4	716.197		
Within Groups	9410.192	35	268.863	2.664	0.049
Total	12274.978	39			

Table IV-21
ANOVA of data for *in vitro* lipid release from the fat body of *I. limbata* incubated with various concentrations of $LaCl_3$ in the presence of native CC extract and calcium

Source	Sum of Squares	df	Mean Square	F	Sig. (P)
Between Groups	6497.865	4	1624.466		
Within Groups	10260.280	30	342.009	4.750	0.004
Total	16758.145	34			

Table IV-23
ANOVA of data for *in vitro* lipid release from the fat body of *I. limbata* incubated with various concentrations of $CdCl_2$ in the presence of native CC extract and calcium

Source	Sum of Squares	df	Mean Square	F	Sig. (P)
Between Groups	4102.126	4	1025.531		
Within Groups	9040.851	30	301.362	3.403	0.021
Total	13142.977	34			

Table IV-25
ANOVA of data for *in vitro* lipid release from the fat body of *I. limbata* incubated with various concentrations of thimerosal in the absence of calcium

Source	Sum of Squares	df	Mean Square	F	Sig. (P)
Between Groups	2560.392	4	640.098		
Within Groups	34668.957	40	866.724	0.739	0.571
Total	37229.349	44			

Chapter 5

Discussion

DISCUSSION

The capacity to regulate the internal ionic composition is a fundamental property of all living organisms (Stewart and Broadus, 1987) including insects. Of the complement of inorganic ions in such organisms, Ca^{2+} performs an especially large number of intracellular and extracellular functions. Calcium ion is involved in the control of a plethora of physiological processes in insects including muscle contraction, neurotransmitter release, gene expression and signal transduction of neurohormones and is found to be the major second messenger for several insect neuropeptides. Calcium has also been found to serve as an important extracellular first messenger (Brown *et al*., 1995).

The levels of both intracellular and extracellular Ca^{2+} found to modify various physiological activities of the cells. Two major sources contribute to the elevation of intracellular Ca^{2+} level: ER and extracellular space, the filling state of the former being dependent on the latter (Walker and Waard, 1998). Thus, $Ca^{2+}{}_{o}$ store provides sufficient Ca^{2+} for the intracellular activities and thereby mediates and modulates several physiological activities of both excitable and non-excitable cells. During the signal transduction of calcium dependent insect neuropeptides in general, Ca^{2+} is found to have both intracellular and extracellular roles to play in modifying the hormone action.

The experiments of the present investigation were aimed mainly at elucidating the role of calcium in the release of lipids from the fat body of

I. limbata both *in vitro* and *in vivo*. Lipid release induced by the synthetic adipokinetic neuropeptide, Lom-AKH-I and crude native CC extract were studied in the presence and in the absence of Ca^{2+}_{o}. Experiments were also carried out to find the mode of action of Ca^{2+} employing calcium ionophore, A23187, calcium channel blockers, lanthanum and cadmium, calcium chelator, EGTA and internal calcium mobiliser, thimerosal on lipid release.

From all the above mentioned experiments, it was seen that lipid release from the fat body of *I. limbata* was influenced by the presence of ionic calcium in the extracellular medium to a great extent and also by calcium originating from intracellular sources. These results thus conform to the general pattern of calcium activity in signal transduction of higher animals (Shima *et al*., 1978; Kojima *et al*., 1985; Jamaluddin *et al*., 1989; Johnson and Chang, 2000).

The results obtained from the experiments *in vitro* using calcium chloride strongly indicate the involvement of Ca^{2+}_{o} on fat body metabolism in *I. limbata*. Here the physiological concentrations of Ca^{2+}_{o} considerably increased the lipid release from the fat body. This effect of Ca^{2+}_{o} might be achieved by the influx of Ca^{2+} into the fat body cells since the effect was found to be blocked by lanthanum (in a dose dependent manner), a non specific calcium channel blocker (Table IV-18). The calcium influx into the fat body cells might be for replenishing the depleted intracellular Ca^{2+} stores (Putney, 1990). It has been shown that the depletion of intracellular Ca^{2+} stores activates the influx of Ca^{2+} into the cells (Putney, 1986 a). The washing of fat body in

the calcium free medium prior to incubation possibly resulted in the partial depletion of internal calcium store and consequently $Ca^{2+}{}_{o}$ entered the cells when the fat body pieces were transferred to Ca^{2+} containing incubation medium. Such depletion of internal Ca^{2+} while washing insect tissues in calcium free buffer was reported earlier (Hayes *et al*., 1995). Further, Keeley *et al.* (1996) reported that equilibration of the fat body of *B. discoidalis* with Ca^{2+} fills internal reservoir. It appears possible that at least some insect cells have a passive inward Ca^{2+} leak pathway, i.e., Ca^{2+} entry in the absence of agonist stimulation as has been reported in the case of PG cells of *M. sexta* (Fellner *et al*., 2005).

The stimulatory effect of Ca^{2+} observed *in vivo,* supported the results from the *in vitro* experiments. However, it should be noted that, in spite of the presence of full complement required for lipid mobilization and release *in vivo*, the extent of lipid release induced by calcium both *in vivo* and *in vitro* conditions appeared almost similar. The experiments further provide indications as to the mode of entry of calcium into the fat body cells *in vivo*, thus giving strong evidence for the presence of a Ca^{2+} leak pathway for Ca^{2+} influx as has been shown in some insects.

Thus, it has been seen that $Ca^{2+}{}_{o}$ mimic the hyperlipaemic action of AKH or octopamine. The mimicking action of $Ca^{2+}{}_{o}$ as calcium-dependent neuropeptides is common among insects. Several authors have demonstrated the AKH peptide mimicking action of $Ca^{2+}{}_{o}$ in the fat body of insects including

mobilization of DAG in *S. gregaria* (Spencer and Candy, 1976), activation of glycogen phosphorylase in *P. americana* (McClure and Steele, 1981), hypertrehalosaemic action in *P. americana* (Steele and Paul, 1985) and in *B. discoidalis* (Park and Keeley, 1995) and inhibition of fatty acid synthesis in *S. gregaria* (Gokuldas, 1989). Extracellular calcium was also found to mimic the action of other insect neuropeptides like allatotropin in *D. punctata* (Kikukawa *et al*., 1987), *L. migratoria* (Dale and Tobe, 1988), *M. sexta* (Allen *et al*., 1992), *G. bimaculatus* (Klein *et al*., 1993) and in *M. loreyi* (Hsieh *et al*., 2002).

Our investigations showed that the effect of $Ca^{2+}{}_{o}$ on lipid release is dose dependent. The inhibitory effect of calcium observed at higher concentrations might be due to the pharmacological effects of calcium on the enzyme systems. Morishima (1979) suggested that as the Ca^{2+} concentration increases within the cell, it would eventually 'turn off' AC when the level becomes inhibitory. Adenylate cyclase catalyses the synthesis of cAMP, that activates a protein kinase which in turn activates lipase. So at higher concentrations of Ca^{2+}, lipid release was low probably due to the inhibition of AC. Regulation of AC activity by Ca^{2+} has been shown in the brain of the moths *M. configurata* and *M. sexta* where lower concentrations of Ca^{2+} stimulate and higher concentrations inhibit AC (Bodnaryk, 1983; Combest *et al*., 1985). Inhibition of AC by higher concentrations of Ca^{2+} is probably due to the known effect of Ca^{2+} as a competitive inhibitor of Mg^{2+} (Garbers and

Johnson, 1975). It is also possible that precipitation of ATP by higher concentrations of Ca^{2+} (Clapham, 1995) leads to a reduced level of cAMP synthesis. From the above observation, it is reasonable to suggest that cAMP may also be involved in the lipid mobilization from the fat body of *I. limbata*.

The hormones belonging to the AKH/RPCH family generally show certain degree of cross reactivity. For example, Lom-AKH-I brought about colour change in the crustacean, *P. borealis* (Fernlund, 1974), hypertrehalosaemia in cockroaches (Holwerda *et al*., 1977), lipid mobilization in *M. sexta* (Shapiro and Law, 1983) and stimulation of locomotory activity in *Pyrrhocoris apterus* (Socha *et al*., 1999). The cross reactivity of synthetic Lom AKH-I was also established in *I. limbata* (Rasheed and Gokuldas, 2002) where Lom-AKH-I was shown to induce hyperlipaemia. The cross reactivity of AKH peptides is possibly due to the sequence homology of the various peptides (see Goldsworthy *et al*., 1997 and Table II-1) and also of their respective receptors. But the actual mechanism of signal transduction in such cross reactivity of AKH is not well understood.

Extracellular calcium is found to modulate AKH induced mobilization of food reserves from the fat body of different species of insects. The mobilization and release of carbohydrate from the fat body of locusts (Vroemen *et al*., 1995 a), cockroaches (Keeley *et al*., 1996; Becker *et al*., 1998; Steele and Ireland, 1999) and fruit beetles (Auerswald and Gäde, 2001 b) by the AKH depend on the availability of calcium. Similarly, lipid

mobilization by AKH from the fat body of locusts (Spencer and Candy, 1976) and moths (Arrese *et al*., 1999) are also calcium dependent. Furthermore, calcium is also essential for AKH-induced proline synthesis and its release from the fat body of *P. sinuata* (Auerswald and Gäde, 2001 a). The results from the present study provide sufficient evidence for the involvement of Ca^{2+}_{o} in the mobilization and release of lipids from the fat body of *I. limbata* by Lom-AKH-I. It has been shown that cross reactivity of Lom-AKH-I in *I. limbata* is absolutely dependent on the Ca^{2+}_{o}. The stimulatory activity of Lom-AKH-I was completely abolished when the calcium in the medium was either omitted or chelated with EGTA. This complete failure of Lom-AKH-I action in the absence of calcium could be due to its dependence on Ca^{2+} for binding to the membrane receptor. Such dependence on calcium has been demonstrated in the binding of AKH to the fat body receptor in *M. sexta* (Ziegler *et al*., 1995). Vroemen *et al*. (1998) lent support to this result with his observations in *L. migratoria*. Hayes *et al.* (1995) reported similar requirement of Ca^{2+}_{o} for small PTTH action in *M. sexta*.

It appears from the aforesaid that in *I. limbata*, Lom-AKH-I depends on Ca^{2+}_{o} not only for its binding to the receptor but also for initiating or maintaining the signal cascade within the cell. This is possible since the Lom-AKH-I induces the influx of Ca^{2+}_{o} into the target tissue. Peptides of AKH/RPCH family were reported to induce influx of calcium into the fat body cells, for example in *P. americana* (Steele and Paul, 1985; Steele and Ireland,

1999), *L. migratoria* (Wang *et al.*, 1990; Van Marrewijk *et al.*, 1993; Vroemen *et al*., 1995 a) and in *P. sinuata* (Auerswald and Gäde, 2001 b).

In its dependence upon calcium, Lom-AKH-I is similar to other insect neuropeptides like proctolin, which initiates contraction of hindgut muscles in *L. maderae* (Cook *et al*., 1975), trehalogon, which activates phosphorylase in *P. americana* (Steele, 1980), allatostatin, which inhibits JH release in *D. punctata* (Aucoin *et al*., 1987), eclosion hormone, which triggers ecdysis behaviour in silkworm (Shibanaka *et al*., 1994), HrTH, which induces trehalose production in *B. discoidalis* (Keeley and Hesson, 1995) and small and big PTTH, which stimulate ecdysteroidogenesis in *M. sexta* (Hayes *et al*., 1995; Girgenrath and Smith, 1996). Calcium dependency has also been reported in the case of diuretic hormone, which induces myotropic activity in *A. domesticus* (Blake *et al*., 1996), allatotropin, which promotes JH synthesis in *M. loreyi* (Kou and Chen, 2000 a, b), CAP_{2b}, which stimulates fluid transport in MT of *Drosophila* (Mac Pherson *et al*., 2001), CCCP, which induces oviduct contraction in *L. migratoria* (Donini and Lange, 2002) and PBAN, which stimulates pheromone production in *D. punctata* (Zhao *et al*., 2002) and in *H. zea* (Choi and Jurenka, 2004).

The results from our investigations (Figure IV-5) clearly showed that in *I. limbata* the modulating effect of Ca^{2+} on Lom-AKH-I-induced lipid release is dose dependent. This finding is consistent with the observation that most of the AKH peptide-induced actions in insect fat body are dependent on the

amount of Ca^{2+}_{o} available. The activation of phosphorylase in *L. migratoria (*Van Marrewijk *et al*., 1991; Vroemen *et al*., 1995 a) and *P. sinuata* (Auerswald and Gäde, 2001 a), proline synthesis in *P. sinuata* (Auerswald and Gäde, 2001 b) and the activation of PLA_2 in *P. americana* (Sun and Steele, 2002) are examples of AKH actions dependent on the concentration of Ca^{2+}_{o}. In our results, maximum stimulation of lipid release with Lom-AKH-I was attained at a calcium chloride concentration of 1.5 mM. Calcium concentration within a similar range (1.0 to 2.0 mM) was found to be essential for the activation of glycogen phosphorylase in the fat body of *L. migratoria* by Lom-AKH-I, -II and -III (Van Marrewijk *et al*., 1991; Vroemen *et al*., 1995 a) and in the fat body of *P. sinuata* by Mem-CC (Auerswald and Gäde, 2001 a).

Higher concentrations of calcium have inhibitory effects on Lom-AKH-I-mediated lipid release from the fat body of *I. limbata*. This inhibitory effects of calcium on Lom-AKH-I action may be attributed not only to the direct effect Ca^{2+} on AC and ATP, but also to their interference with the binding property of the peptide and lipophorin to their respective receptors. The high concentrations of calcium may inhibit the binding of Lom-AKH-I to the receptor. Ziegler *et al*. (1995) reported that Ca^{2+} is essential for the binding of *Manduca*-AKH to their receptor and 2 mM giving slightly better results than 5 mM.

The full complement of lipophorin and lipid transfer particle required for the transfer of lipids from the fat body to the haemolymph is synthesized

and released into the haemolymph from the fat body itself (Van Heusden *et al*., 1996; Van der Horst *et al*., 1997; Van der Horst, 2003). It was seen that lipophorins are released into the surrounding medium in fairly large quantities during *in vitro* incubation of fat body (Bergman and Chippendale, 1989). Calcium is essential for the binding of lipophorin to the fat body membrane receptor in insects (Tsuchida and Wells, 1990; Lee *et al*., 2003). The ligand-binding activity of all members of the LDLP receptor family depends on the presence of divalent cation and this binding is due to the Ca^{2+}-binding ability of cysteine rich ligand binding repeats, which results in the conformational changes of the receptor proteins (Van Driel *et al*., 1987; Blacklow and Kim, 1996). Although Ca^{2+} was essential for the binding of lipoproteins to their receptors, increasing concentration of Ca^{2+} might have an inhibitory effect on the binding which may adversely affect the lipid release. Similar inhibitory properties of higher concentrations of Ca^{2+} on lipophorin binding to the receptor were reported in *G. mellonella* (Lee *et al*., 2003).

The inhibitory effects of higher concentrations of calcium on Lom-AKH-I-induced lipid release from the fat body of *I. limbata* obtained from our experiments are in agreement with the modulating effect of calcium on other insect neuropeptides like trehalogon in *P. americana* (McClure and Steele, 1981) and PBAN in *H. armigera* (Rafaeli and Gileadi, 1996). However, in insects such as *L. migratoria* (Van Marrewijk *et al*., 1991; Vroemen *et al*., 1995 a) and *P. sinuata* (Auerswald and Gäde, 2001 a, b), higher concentrations

of calcium in the range of those used in our investigation (above 2 mM) still had stimulatory effects on AKH-induced activities.

Combinations of Lom-AKH-I and lower concentrations of calcium have cumulative effects on the lipid mobilization from the fat body of *I. limbata* (Table IV-6 and Table IV-8). This cumulative effect could be due to the involvement of other second messenger(s) in the action of Lom-AKH-I in *I. limbata*, than Ca^{2+}. The involvement of more than one second messenger was reported for the signal transduction of AKH in different insects (see Vroemen *et al.*, 1998; Van der Horst, 2003; Gäde and Auerswald, 2003). Calcium and cAMP are involved in AKH-induced DAG production in the fat body of *L. migratoria* (Wang *et al*., 1990) and *M. sexta* (Arrese *et al*., 1999). Similarly, Ca^{2+} and cAMP are the second messengers of AKH peptide-induced proline synthesis in *P. sinuata* (Auerswald and Gäde, 2001 a) while Ca^{2+} and IP_3 are involved in HrTH-dependent glycogen phosphorylase activation in *B. discoidalis* (Park and Keeley, 1996).

Hyperlipaemic activity of crude CC extract has been reported in the plant bug, *I. limbata* (Rasheed and Gokuldas, 2002). The data obtained from our bioassay studies with the HPLC fractions of an extract of brain-CC-CA complex of *I. limbata* revealed that several fractions were responsible for the hyperlipaemic activity in *I. limbata*. At least four fractions have significant adipokinetic activity among which one fraction showed better activity. The presence of more than one active AKH had been reported in other

heteropterans also. For example, two AKH have been identified in *P. apterus* (Kodrik *et al*., 2000, 2002 b). Investigations on the structure of adipokinetic neuropeptides in heteropteran insects such as the stink bug, *Nezara viridula* (Gäde *et al*., 2003), the water bug, *Notonecta glauca* (Gäde *et al*., 2004), the twig wilter, *Holoptera alata* (Gäde *et al*., 2006), the water boatman, *Corixa punctata*, the saucer bug, *Ilyocoris cimicoides* (Gäde *et al*., 2007 a), the giant water bug, *Lethoserus indicus* and the water scorpion, *Nepa cinerea* (Gäde *et al*., 2007 b) are currently underway.

Since adipokinetic signal transduction of Lom-AKH-I appears to be dependent on the Ca^{2+}_{o} in *I. limbata* to a great extent, the signal transduction of endogenous adipokinetic peptides of brain-CC-CA complex may also depend on the presence of calcium. The current results demonstrate that just like Lom-AKH-I, native CC extract also requires Ca^{2+}_{o} for lipid mobilization and release from the fat body. This is evident from the fact that either the omission of calcium or chelation of calcium with EGTA results in the failure of CC extract to induce lipid release from the fat body of *I. limbata* (Table IV-11 and Table IV-14). Similar requirement of Ca^{2+} was established earlier for CC extract-induced actions in insects like lipid mobilization from the fat body of *L. migratoria* (Spencer and Candy, 1976), lipid synthesis inhibition in *G. morsitans* (Pimley, 1985), activation of glycogen phosphorylase in (Orr *et al*., 1985) and efflux of trehalose from (Steele and Paul, 1985) the fat body of *P. americana*.

In our investigation, the CC extract was shown to elicit hyperlipaemia *in vitro* and was seen to be calcium dose-dependent. Dose-response curve of calcium with respect to CC extract-mediated as well as Lom-AKH-I-mediated actions in *I. limbata* were found to be similar and comparable (see Figure IV-4, Figure IV-5 and Figure IV-9). Similarity in the modulating effects of Ca^{2+} on lipid mobilization by Lom-AKH-I and CC extract, is an indication of the existence of structural and functional similarity between Lom-AKH-I and the active components of CC extract. The results also showed that maximum response (44% increase in lipid release) with 1 gpe of CC extract occurred at a $Ca^{2+}{}_{o}$ concentration of 1.5 mM. In the case of Lom-AKH-I (100 pmol) also, maximum activation (34% increase in lipid release) occurred in the presence of 1.5 mM calcium. However, with the other lower concentrations of calcium (1.0 and 2.0 mM), the value obtained with Lom-AKH-I was slightly lower than that obtained with CC extract. This decrease in lipid release may be either due to the difference in the doses of Lom-AKH-I and endogenous neuropeptides of CC extract that are required for complete binding of receptors with peptides or due to the difference in the binding property of the neuropeptides caused due to the difference in amino acid sequence.

The critical role of Ca^{2+} in Lom-AKH-I- and CC extract-mediated mobilization and release of lipids suggests that Ca^{2+} could be acting as a second messenger that mediates the lipid releasing actions of insect neuropeptides in *I. limbata*. If so, an influx of calcium into the fat body cells

might definitely mimic the action of Lom-AKH-I/CC extract. This hypothesis was, therefore, tested by introducing Ca^{2+} into fat body cells of *I. limbata* using the calcium ionophore, A23187. In the present study, introduction of Ca^{2+} by A23187 resulted in a significant stimulation of lipid release from the fat body. Here, lipid release increased by 56% (A23187, 15 µM) over the control in a medium containing 1 mM calcium chloride. This value is higher than what is achieved with 100 pmol of Lom-AKH-I or 1 gpe of CC extract in the presence of calcium. Extracellular Ca^{2+} dependency of the action of A23187 was evident from the fact that ionophore-mediated lipid release was abolished in the absence of calcium. Our results thus demonstrate that the major factor responsible for the stimulation of lipid mobilization and release from the fat body of *I. limbata* is the increase in the concentration of cytoslic calcium. Further, the data presented in Table IV-17 shows that a combination of Lom-AKH-I and A23187 in the calcium containing incubation medium resulted in an activation higher than that obtained with Lom-AKH-I and Ca^{2+} combined. Similarly, a combination of CC extract, A23187 and calcium was also showing better effect, than the effect obtained with CC extract and calcium. These results indicate the importance of influx of $Ca^{2+}{}_{o}$ for neuropeptide induced lipid mobilization and release in *I. limbata*. CC extract- and AKH peptide-induced influx of $Ca^{2+}{}_{o}$ into the fat body has already been demonstrated in insects such as *P. americana* ((Steele and Paul, 1985; Steele and Ireland, 1999), *L. migratoria* (Wang *et al.*, 1990; Van Marrewijk *et al.*, 1993; Vroemen *et al*., 1995 a) and *P. sinuata* (Auerswald and Gäde, 2001 b).

A23187-facilitated Ca^{2+} movement have been reported to simulate the actions of insect neuropeptides such as PTTH in *M. sexta* (Smith *et al*., 1985; Smith and Gilbert, 1986) and *B. mori* (Gu *et al*., 1998), AKH in *L. migratoria* (Van Marrewijk *et al*., 1991) and PBAN in *O. nubilalis* (Ma and Roelofs, 1995).

The importance of the influx of Ca^{2+}_{o} in CC extract-mediated lipid release from the fat body of *I. limbata* was further investigated using lanthanum. Lanthanum (2 mM) inhibited CC extract-induced lipid release in *I. limbata* to 63% of the control. Lanthanum has a high affinity for specific cation binding sites on cell membrane. By virtue of an ionic radius similar to Ca^{2+} and a higher valence than Ca^{2+}, La^{3+} will bind at superficially located Ca^{2+} sites in a less reversible manner than does Ca^{2+}, thereby preventing binding and subsequent entry of Ca^{2+} into cytosol (Weiss, 1974). The inhibition of the effects of CC extract by La^{3+} indicates that the extract acted on the plasma membrane to cause the opening of calcium channels to permit Ca^{2+}_{o} entry. Similarly, cadmium also inhibited CC extract-induced lipid release from the fat body of *I. limbata* almost as effectively as that of lanthanum. These results together with the findings from A23187 treatment strongly suggest that Ca^{2+} act as the major second messenger in the Lom-AKH-I and CC extract-mediated lipid release in *I. limbata*.

The importance of Ca^{2+}_{i}, derived from intracellular stores in Lom-AKH-I/CC extract-mediated lipid release was studied using thimerosal in the absence of Ca^{2+}_{o}. Thimerosal elicited a dose dependent activation of lipid

release. Maximum stimulation to generate a 40% increase in lipid release was noticed at a thimerosal concentration of 100 µM, above and below which the stimulatory effect was lower. The data suggest that thimerosal-induced lipid release may result from an increase in the concentration of cytosolic calcium released from internal stores and that, this lipid release from the fat body was dependent upon an optimal intracellular concentration of calcium. But the stimulation caused by thimerosal was much below the level of activation caused by A23187, Lom-AKH-I and CC extract of *I. limbata*. This could probably be due to the partial depletion of internal calcium store, resulted from the washing of fat body in a Ca^{2+}-free incubation buffer before treatment with thimerosal. The high level of variation between the different samples of the fat body to thimerosal may be explained in terms of the possible difference in the level of depletion of calcium store during washing. Thus it is evident that, in addition to the $Ca^{2+}{}_{o}$, calcium released from intracellular calcium stores is also important in insect neuropeptide-induced lipid release in *I. limbata*. It also appears that the $Ca^{2+}{}_{o}$ is more important than the $Ca^{2+}{}_{i}$.

The fact that thimerosal (which can release Ca^{2+} from IP_3-sensitive internal Ca^{2+} store) can induce lipid release from the fat body of *I. limbata* like Lom-AKH-I and CC extract, indirectly shows that IP_3 may be a part of the second messenger system in the Lom-AKH-I and CC extract-mediated lipid release. The involvement of IP_3 in AKH-induced lipid mobilization has been reported earlier in *L. migratoria* (Vroemen *et al*., 1998; Van der Horst, 2003).

But Gäde and Auerswald (2003) reported that even though Ca^{2+} from IP_3-sensitive intracellular stores are involved in AKH-induced hypertrehalosaemia in different insects, Ca^{2+} from such stores are not a part of AKH-induced hyperlipaemia. However, there have been reports of IP_3-insensitive intracellular Ca^{2+} stores taking part in adipokinetic signalling processes in insects (Arrese *et al*., 1999).

Thus from the present investigation, it is evident that Ca^{2+} acts as the major second messenger in the insect neuropeptide-induced lipid release from the fat body of *I. limbata*. Both extracellular and intracellular Ca^{2+} are involved in this lipid release. It is reasonable to presume that $Ca^{2+}{}_o$ is involved in the binding of the neuropeptide and lipophorin to their respective receptors which are essential for the induction of lipid moblisation and release from the fat body. Internally, Ca^{2+} derived from both intracellular store and extracellular space might be involved in the activation of AC, which is a prerequisite for the activation of TAG lipase, the key enzyme involved in the mobilsation of lipids from the fat body. However, an increase in cytsolic Ca^{2+} may not account for the entire adipokinetic activity *in vivo* in energy requiring physiological and physical activities in *I. limbata*. AKH-dependent second messengers like cAMP and IP_3 may also be involved.

The foregoing account discussed the roles played by calcium in modulating the actions of adipokinetic hormones in regulating lipid mobilization and release from the fat body into the haemolymph or surrounding

medium under various conditions of different experimental combinations. It has been possible to show that calcium is probably the only or the major signal transducing element. Data obtained from the different experiments were subjected to suitable statistical analyses for testing the significance of the effects. It was found that inspite of the variations observed, most of the effects were significant and reproducible. In insects, variations are expected as the individual insects usually exhibit high degree variations as to their physiological status, feeding status, sex and age. Maximum attempts were made to avoid these variations by taking controls and experiments from the same set of insects, one half of the fat body serving as the controls and the other half as the experimentals. Insects were taken from a colony maintained on uniform dietary and photoperiodic conditions. The variation between and among the different groups could still be minimised by maintaining uniformity with regard to various parameters mentioned above.

Further detailed investigations are required in this line to find out

1. The amino acid sequence of the peptides of CC extract of *I. limbata* which are responsible for hyperlipaemia.
2. The mechanism of hormone-induced influx of Ca^{2+}_{o} into the fat body cells.
3. Further evidence for the involvement of cAMP and IP_3 as the second messengers of adipokinetic signalling in *I. limbata.*

Summary

SUMMARY

Fractionation of crude extract of brain-CC-CA complex of *Iphita limbata* using HPLC showed that the tissue complex contained several factors of peptidic nature. This was evident from the presence of a large number of (major and minor) peaks. On *in vivo* bioassays of the different HPLC fractions, it was found that, of all the factors having hyperlipaemic activity, significant activity was restricted only to four fractions

Calcium ion seems to be the major second messenger in the adipokinetic activity of the insect neuropeptides in *I. limbata*. Both extra- and intracellular Ca^{2+} are involved in their actions. The hyperlipaemic effects of both crude CC extract of *I. limbata* (with adipokinetic neuropeptides as evidenced from HPLC fractionation followed by bioassays with HPLC fractions) and synthetic Lom-AKH-I on the fat body of *I. limbata* were totally dependent on the presence of calcium in the incubation medium. The stimulatory effects of these adipokinetic factors were totally abolished when calcium in the medium were omitted or chelated with calcium specific chelator, EGTA.

Extracellular Ca^{2+} was found to modulate the effects of both crude CC extract and Lom-AKH-I in a dose dependent manner. Lower concentrations of calcium (1.0, 1.5 and 2.0 mM) had stimulatory effects. However, higher concentrations of calcium (4 and 8 mM) considerably suppressed this

hyperlipaemic effect. Extracellular Ca^{2+} is essential for the binding of adipokinetic peptides to their respective receptors.

Evidence for the involvement of Ca^{2+} as a second messenger in the action of insect neurpeptides came from the observation that an influx of Ca^{2+} assisted by A23187 into the fat body mimicked the actions of adipokinetic neuropeptides. In addition, CC extract-induced lipid release was considerably inhibited by calcium channel blockers, La^{3+} and Cd^{2+} which would block Ca^{2+} influx into the fat body cells. A thiol compound, thimerosal which can release calcium from IP_3-sensitive calcium stores could mimic the adipokinetic actions of crude CC extract and synthetic Lom-AKH-I indicate that Ca^{2+} from intracellular stores is also involved in the adipokinetic activity of neuropeptides in *I. limbata*. Results from the present investigations provide clear indications for the involvement of cAMP and IP_3 in the signal transduction of the adipokinetic peptides in *I. limbata*.

Furthermore, the present study also showed that lower concentrations of calcium (1.0, 1.5 and 2.0 mM) stimulate the fat body to elevate the basal level of lipid release significantly *in vivo* and *in vitro* even in the absence of lipid mobilizing agents, whereas higher concentrations (4.0 and 8.0 mM) have inhibitory effect on lipid release. Calcium induced lipid release was considerably decreased by the non-specific calcium channel blocker, La^{3+}.

In conclusion, the investigations showed that lipid mobilization from the fat of *I. limbata* is regulated by the level of extracellular and intracellular Ca^{2+}.

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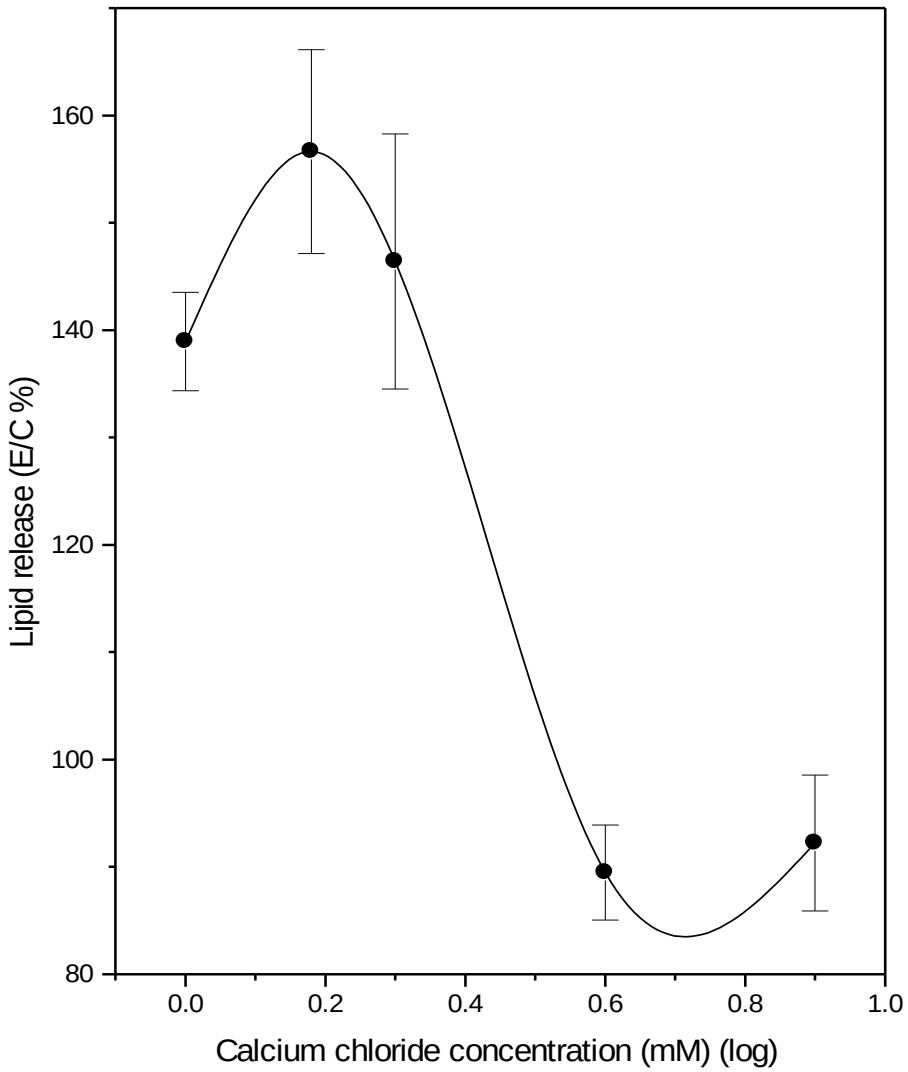


Figure IV-5. Combined effect of Lom-AKH-I and calcium on lipid release from the fat body of *I. limbata*. Fat bodies were incubated in HEPES buffer containing 100 pmol of Lom-AKH-I and indicated concentrations of $CaCl_2$; controls lacked $CaCl_2$ and Lom-AKH-I. Each point represents mean and vertical line indicates SEM (n=9).

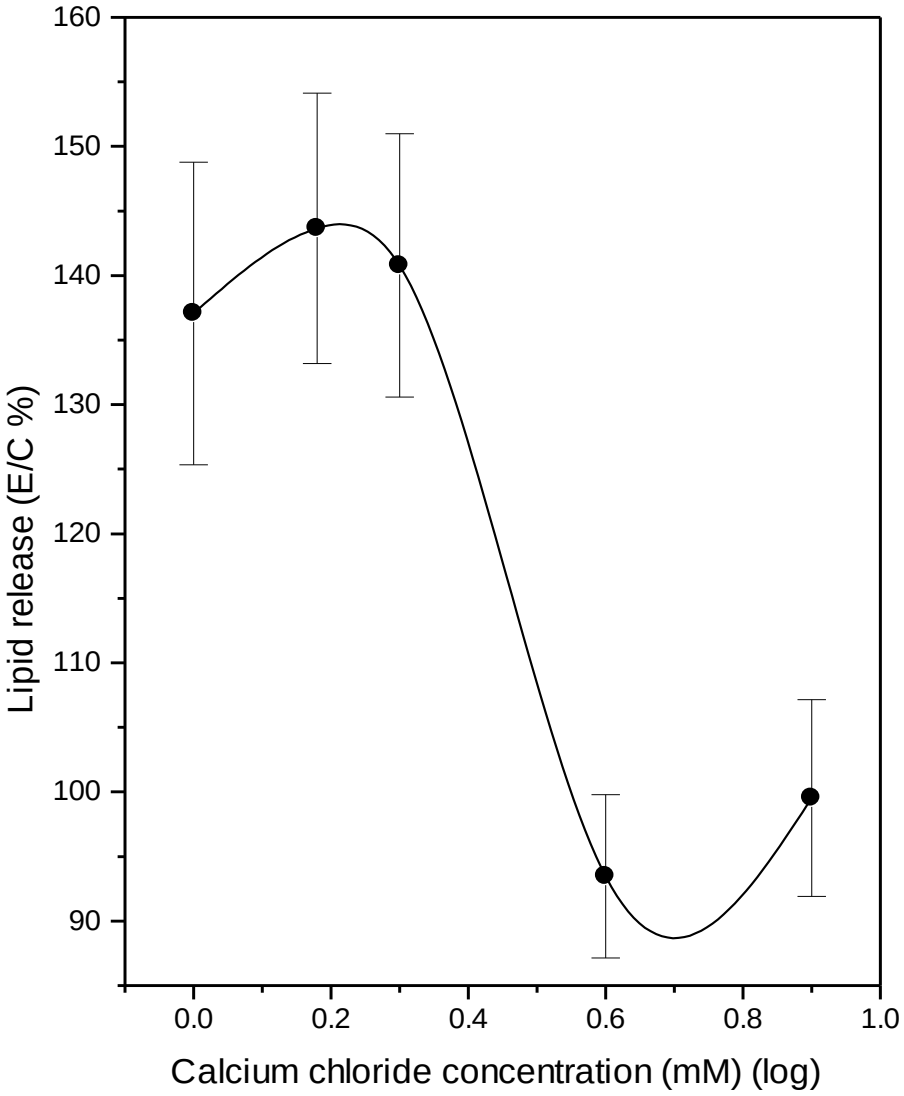


Figure IV-9. Effect of CC extract on lipid release from the fat body of *I. limbata* in the presence of various concentrations of $CaCl_2$. Fat body pieces were incubated in HEPES buffer containing indicated concentrations of calcium and 1 gpe of CC extract; controls lacked CC extract. Each point represents mean and vertical line indicates SEM (n=9).

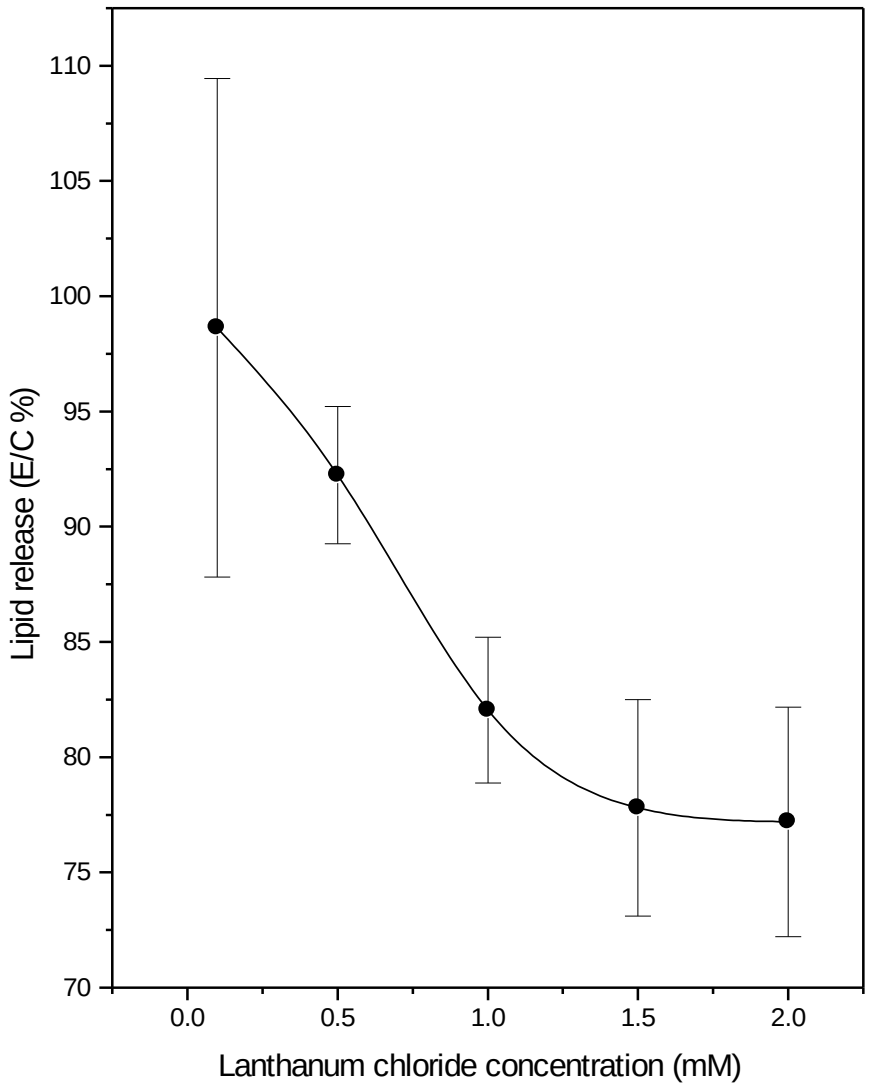


Figure IV-11. Effect of lanthanum on Ca^{2+}-induced lipid release from the fat body of *I. limbata*. Fat body pieces were incubated in calcium (1 mM) containing medium with (experimental) or without (control) $LaCl_3$. Each point represents mean and vertical line indicates SEM (n=8).

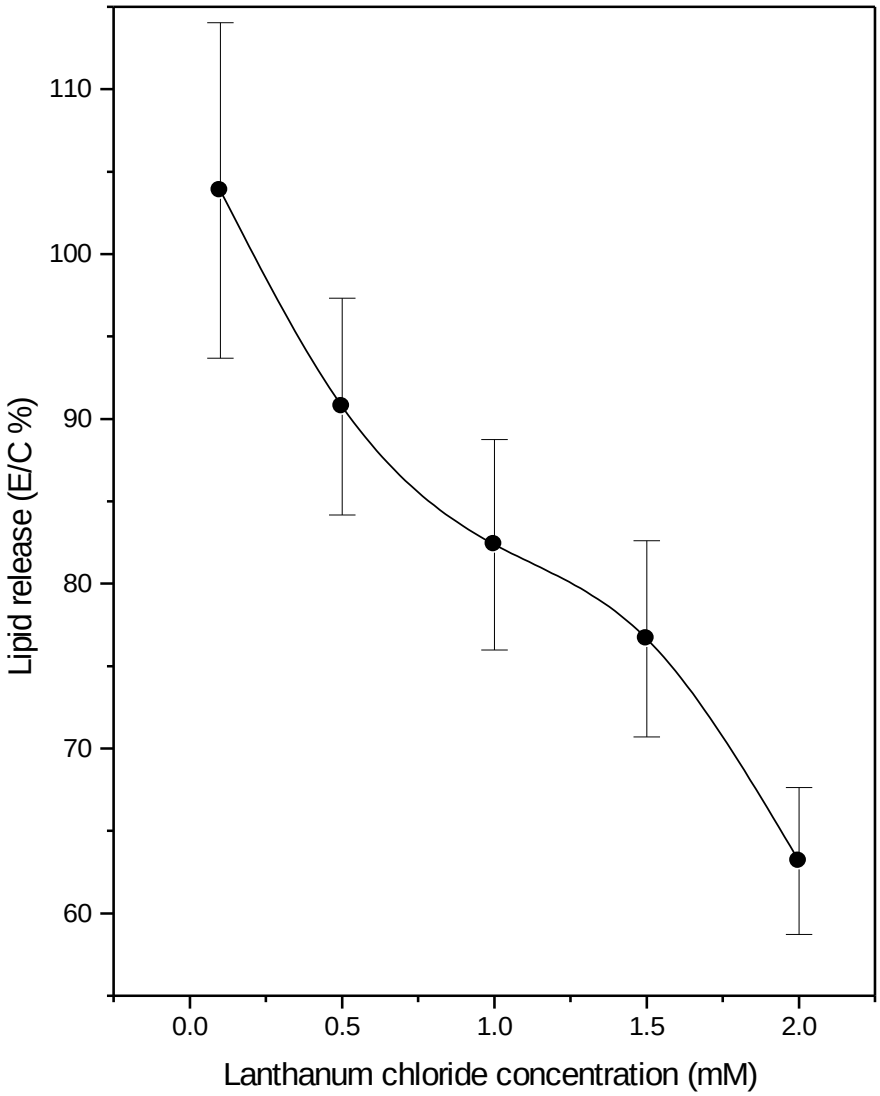


Figure IV-12. Effect of lanthanum on CC extract-mediated lipid release from the fat body of *I. limbata* in the presence of extracellular Ca^{2+}. For experimental, fat body was incubated in Ca^{2+} (1 mM) containing medium in the presence of CC extract (1 gpe) at various concentrations of $LaCl_3$; control incubation was without $LaCl_3$ but with CC extract and Ca^{2+}. Each point represents mean and vertical line indicates SEM (n=7)

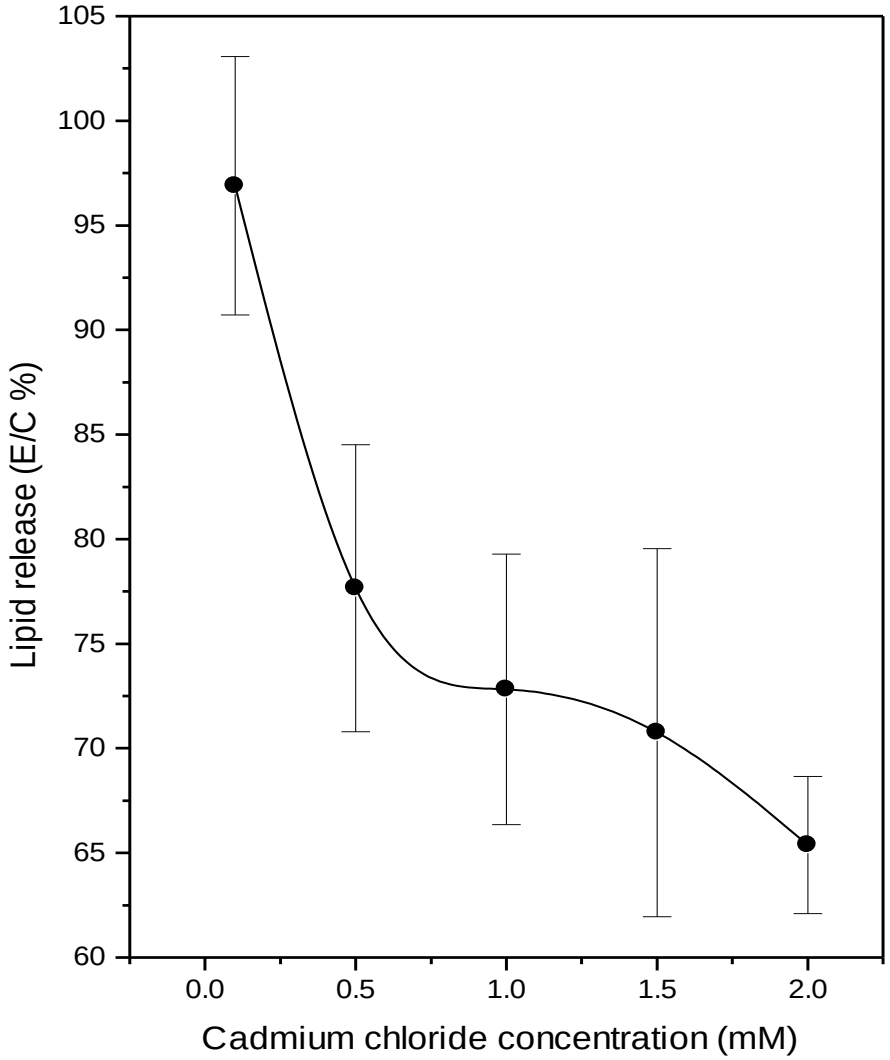


Figure IV-13. Effect of cadmium on CC extract-mediated lipid release from the fat body of *I. limbata* in the presence of extracellular Ca^{2+}. For experimental, fat body was incubated in Ca^{2+} (1 mM) containing medium in the presence of CC extract (1 gpe) at various concentrations of $CdCl_2$; control incubation was without $CdCl_2$ but with CC extract and Ca^{2+}. Each point represents mean and vertical line indicates SEM (n=7).

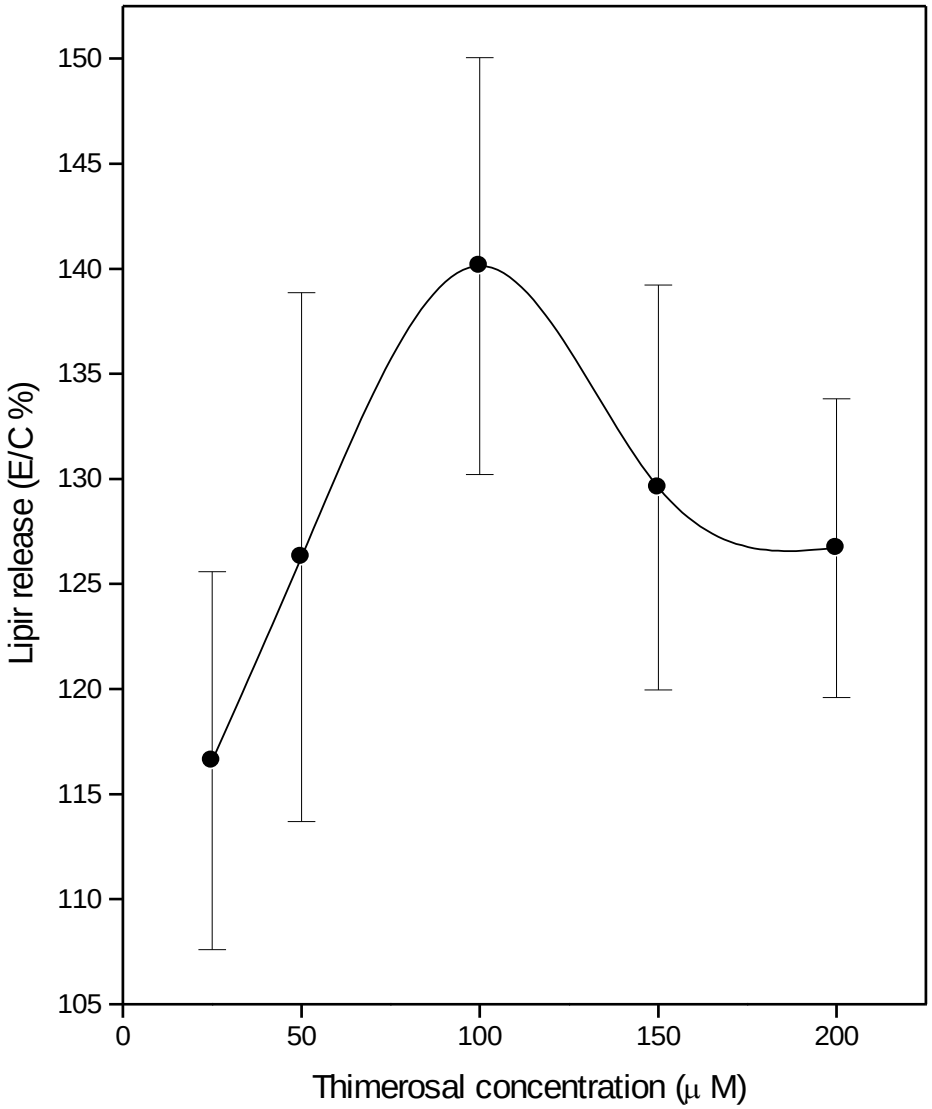


Figure IV-14. Effect of thimerosal on lipid release from the fat body of *I. limbata*. Fat body pieces were incubated in Ca^{2+}-free HEPES buffer either in the presence of indicated concentrations of thimerosal (experimental) or in the absence of thimerosal (control). Each point represents mean and vertical line indicates SEM (n=9).

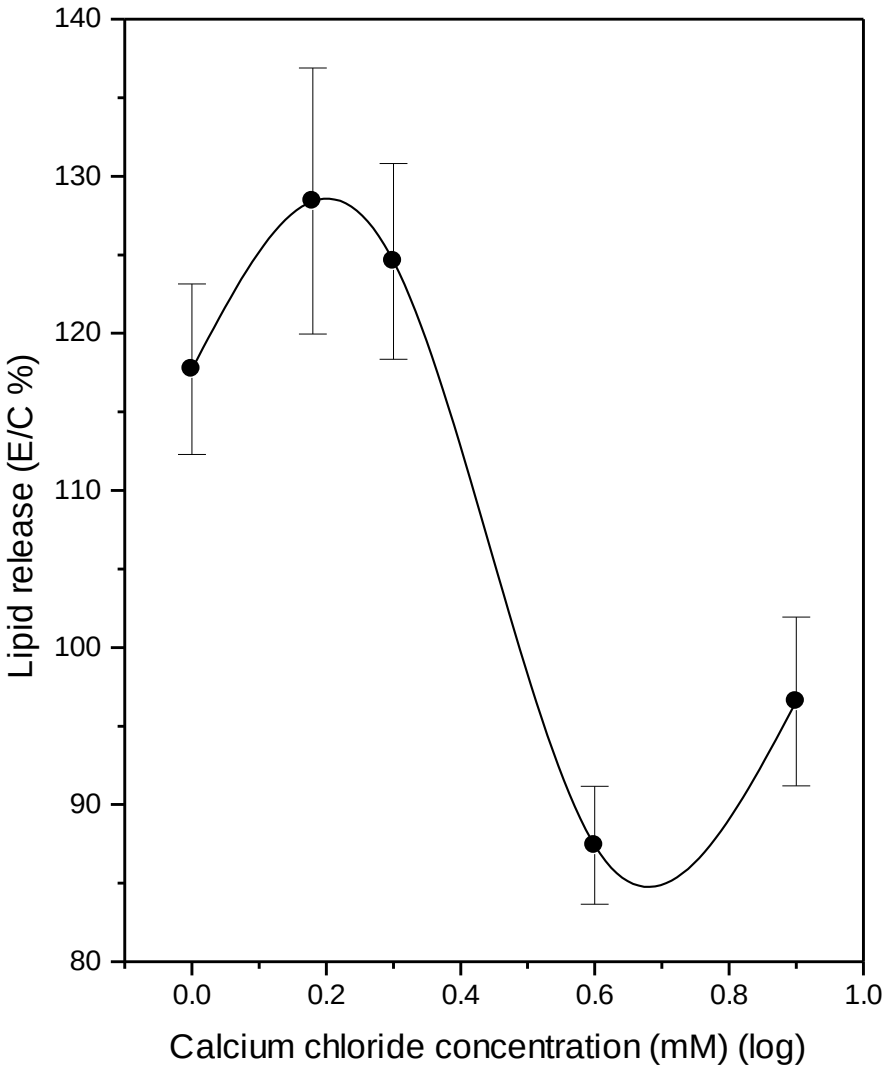


Figure IV-1. Effect of extracellular Ca^{2+} on lipid release from the fat body of *I. limbata in vitro*. Fat body was incubated for 35 min in the absence or in the presence of $CaCl_2$ with different concentrations as indicated. Each point represents mean and vertical line indicates SEM (n=9).

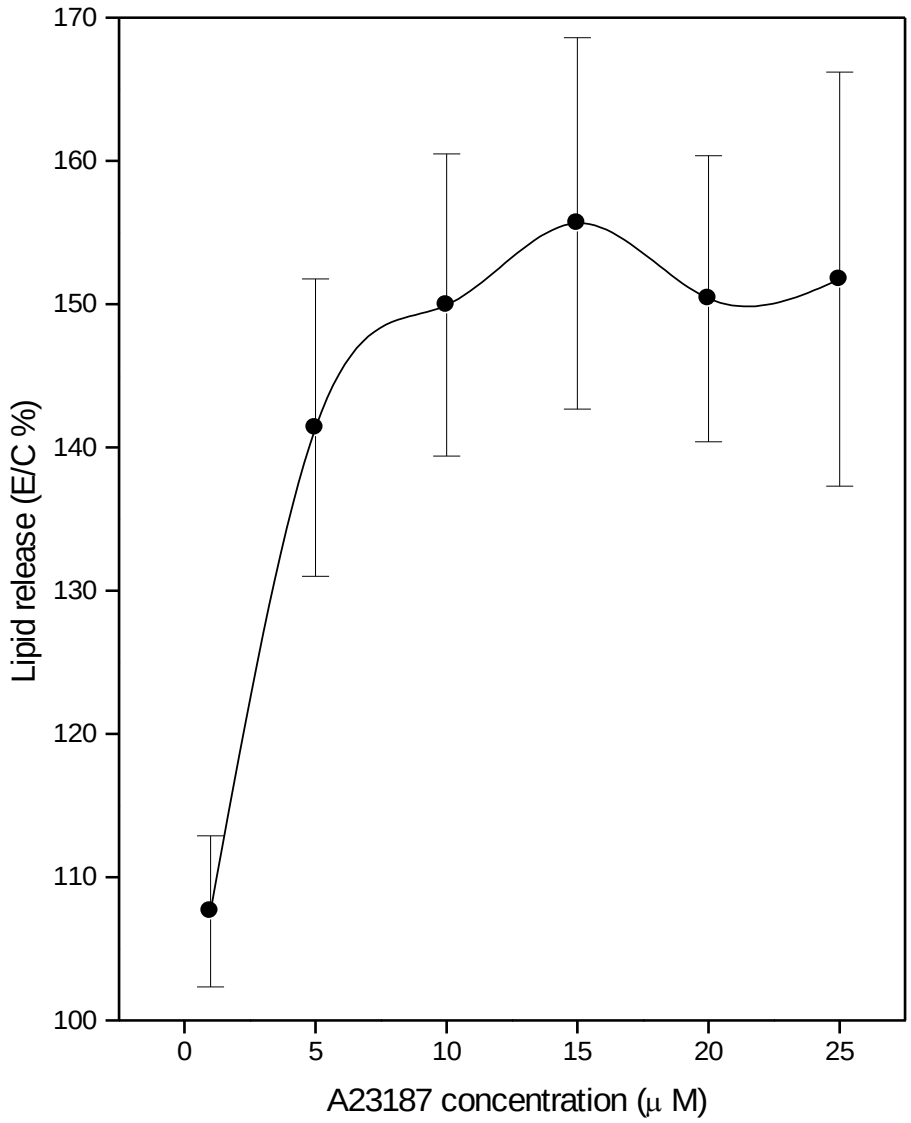


Figure IV-10. Dose response of A23187 on lipid release from the fat body of *I. limbata* in the presence of Ca^{2+}. Fat body was incubated in calcium (1 mM) containing HEPES buffer with indicated concentra-tions of A23187; controls lacked A23187. Each point represents mean and vertical line indicates SEM (n=9).

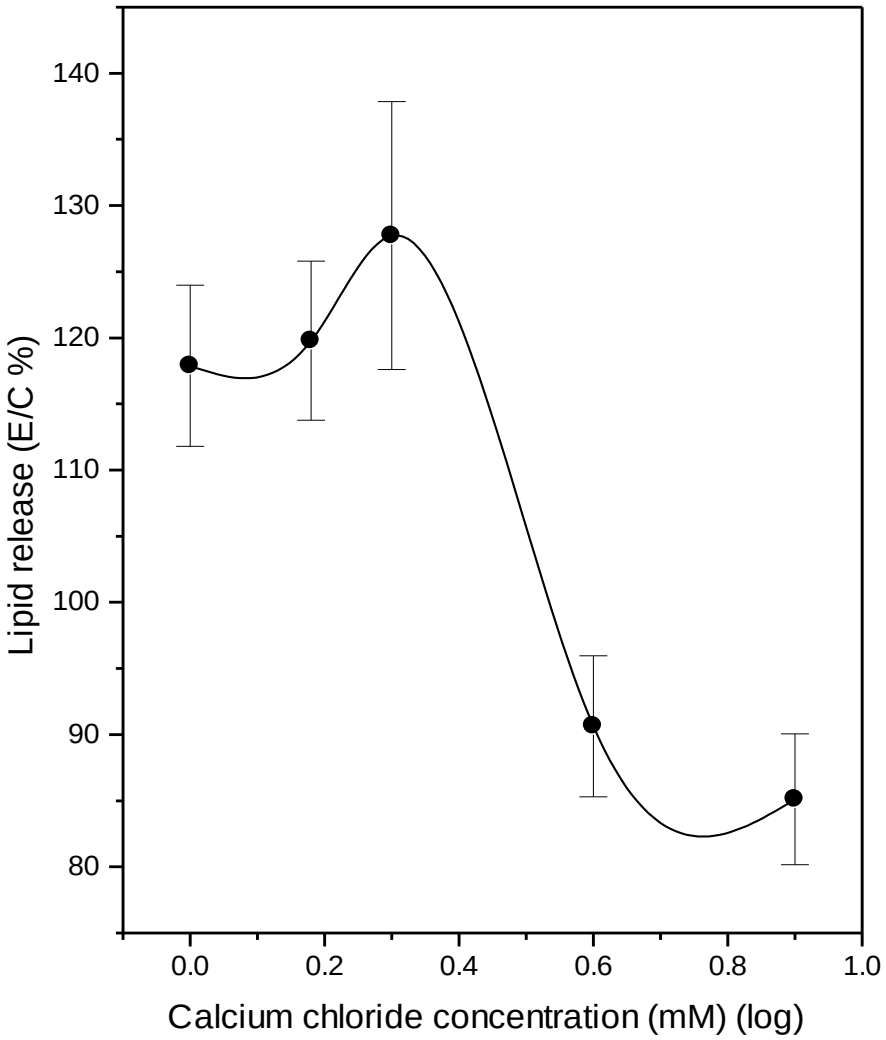


Figure IV-2. Effect of extracellular Ca^{2+} on lipid release from the fat body of *I. limbata in vivo*. Insects were injected with various concentrations of $CaCl_2$. Haemolymph samples were collected before and 35 min after injection for lipid estimation. Each point represents mean and vertical line indicates SEM (n=12).

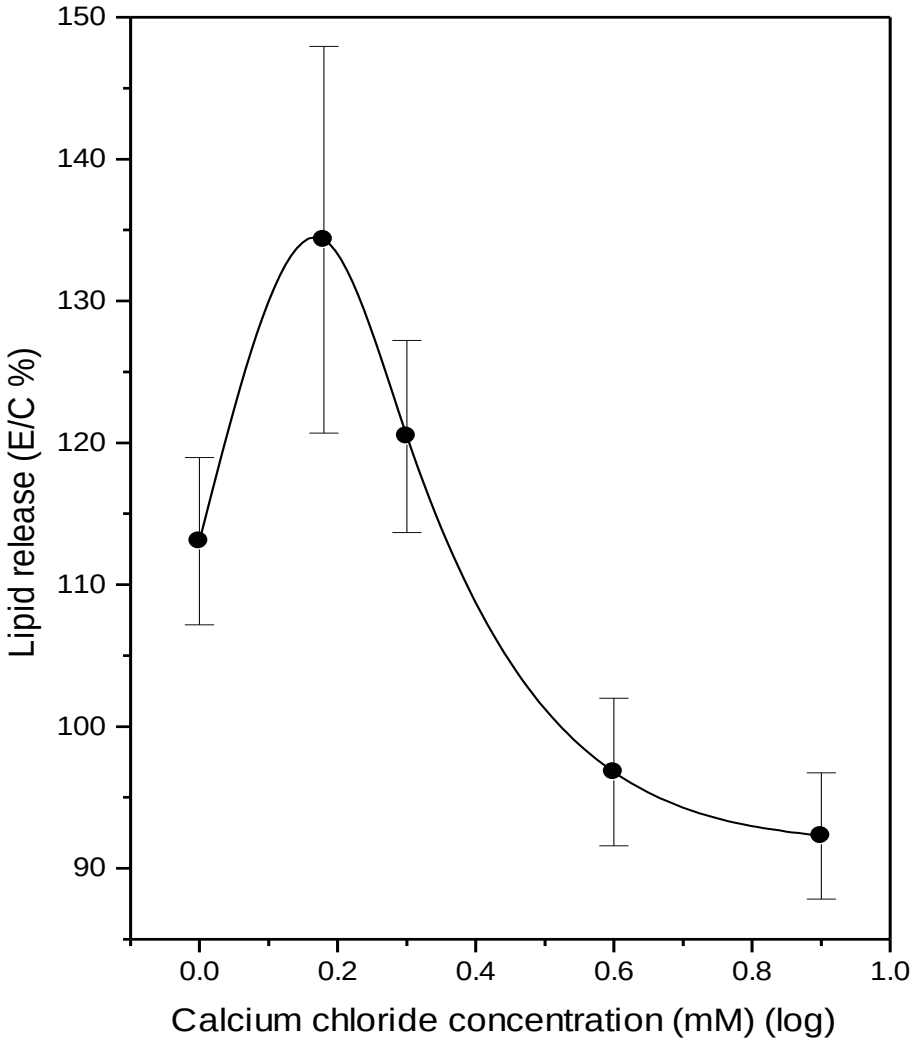


Figure IV-4. Effect of Ca^{2+} on the lipid releasing action of Lom-AKH-I on the fat body of *I. limbata*. The fat body was incubated in a Ca^{2+} containing buffer with or without Lom-AKH-I (100 pmol/ incubation). Each point represents mean and vertical line indicates SEM (n=9).

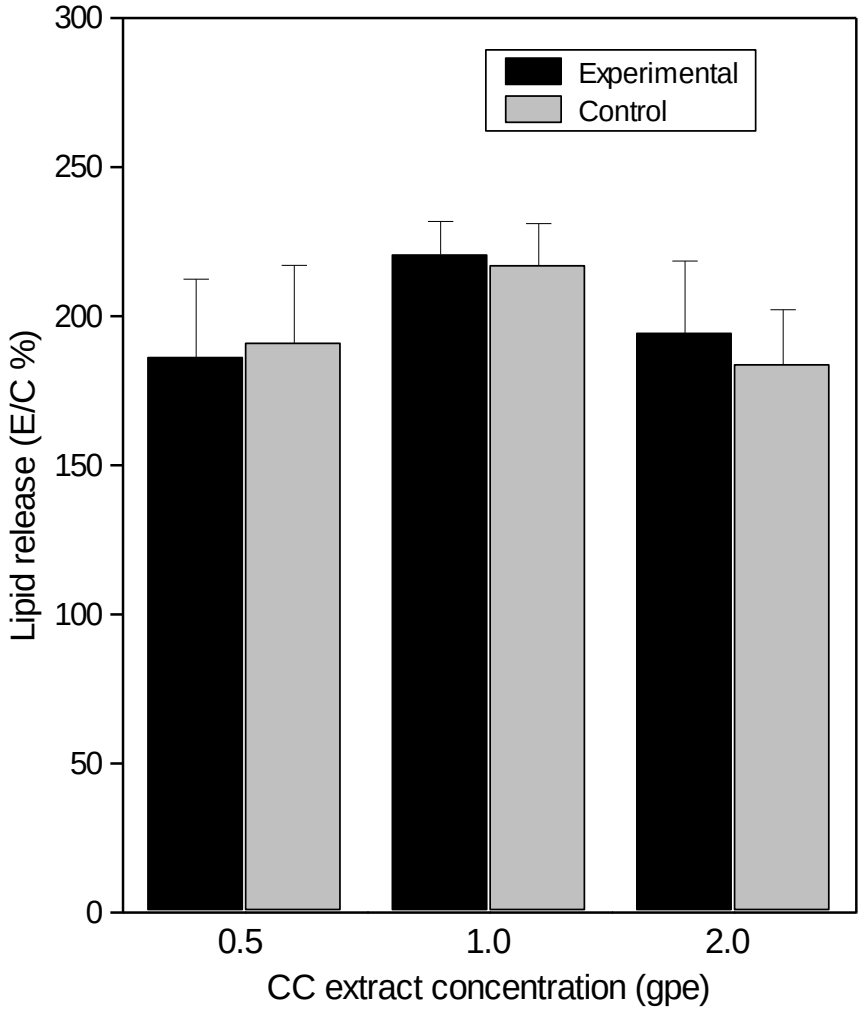


Figure IV-8. Effect of crude native CC extract on lipid release from the fat body of *I. limbata* in the absence of extracellular Ca^{2+}. Fat body was incubated in a Ca^{2+}-free medium with (experimental) and without (control) CC extract. Bars represent means ± SEMs (n=7).

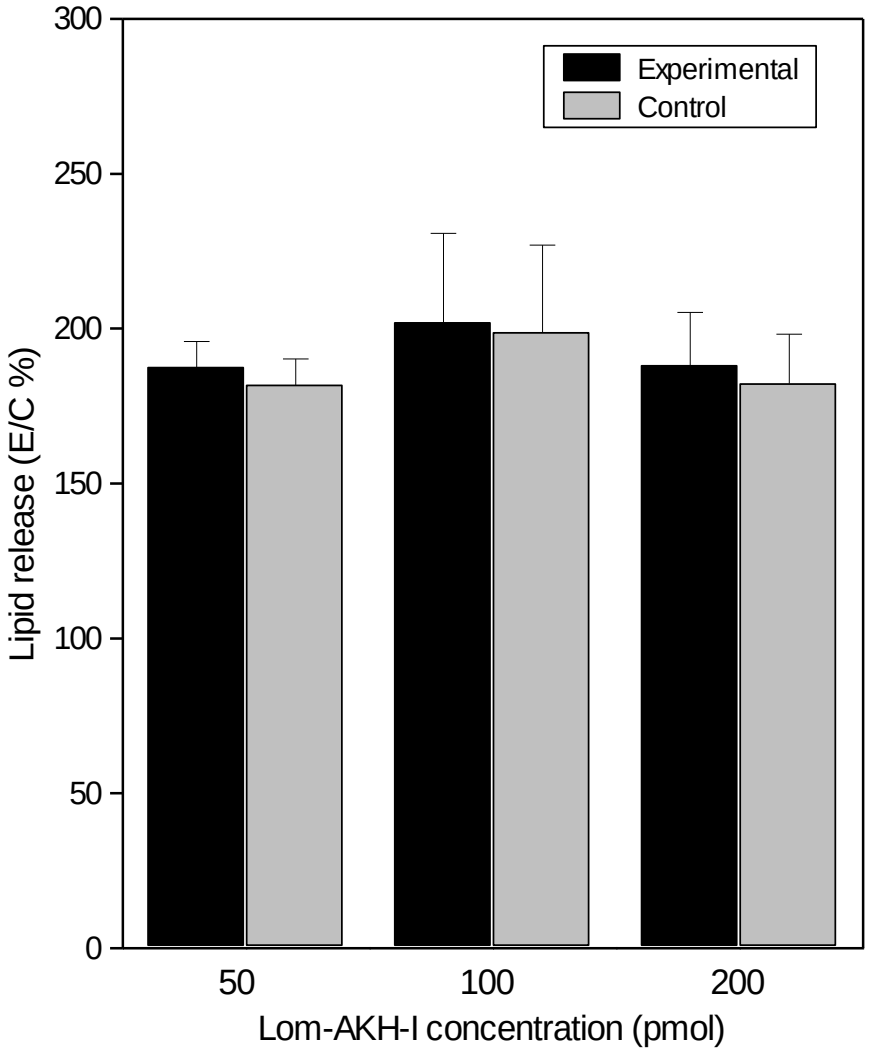


Figure IV-3. Effect of Lom-AKH-I on lipid release from the fat body of *I. limbata* in the absence of extracellular Ca^{2+}. The fat body was incubated in a Ca^{2+}-free buffer with (experimental) and without (control) Lom-AKH-I. Bars represent means ± SEMs (n=7).

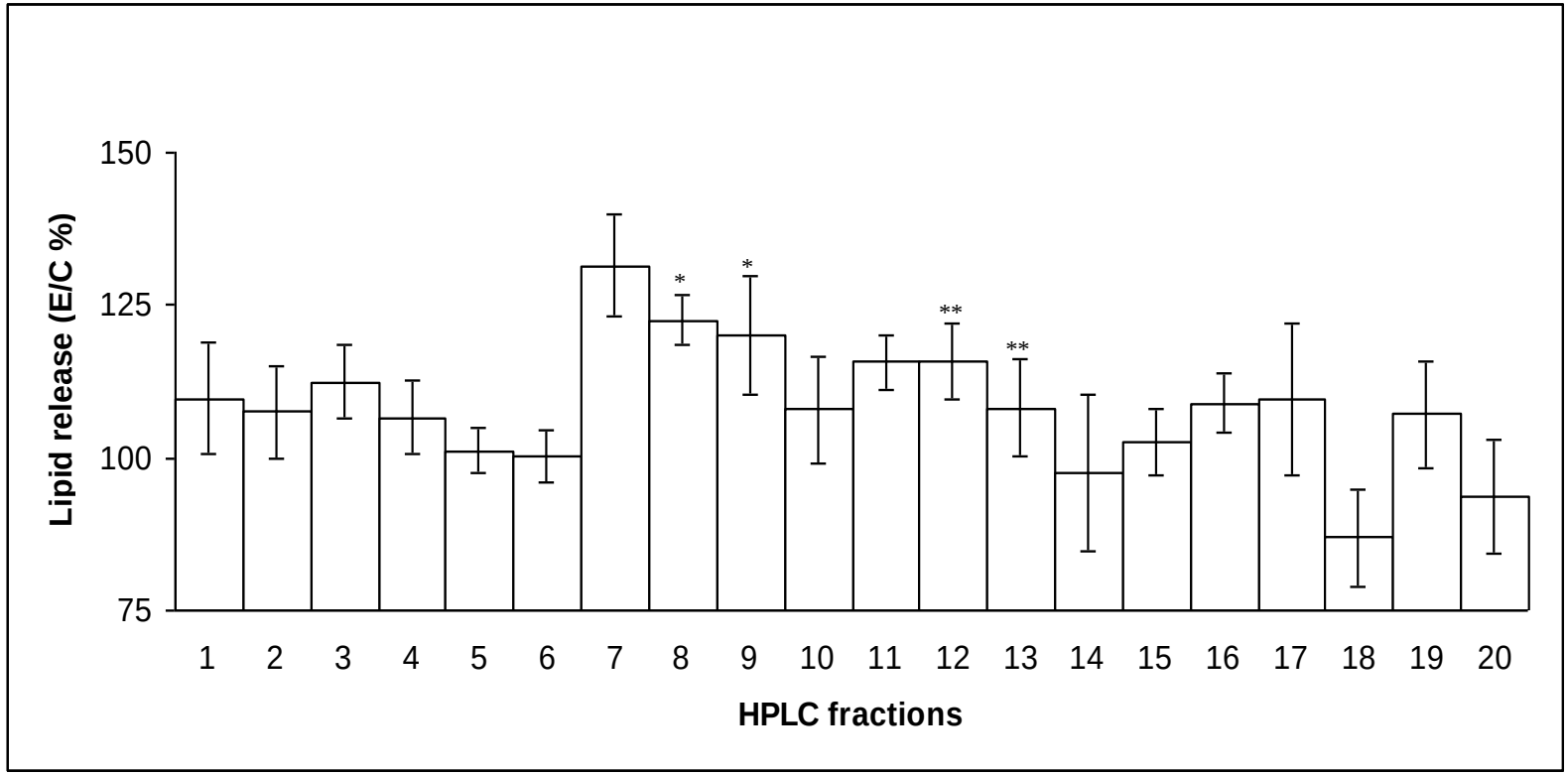
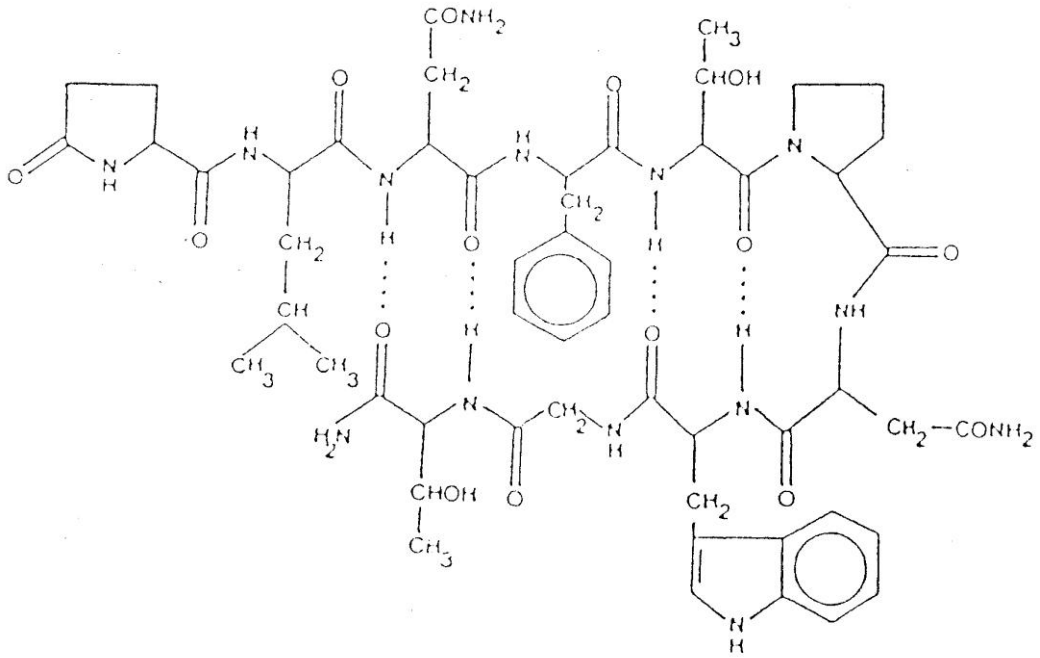


Figure IV-7. Adipokinetic activity of HPLC fractions of the extract of brain-CC-CA complex of *I. limbata*. Fractions were collected from an extract of 40 tissue complexes. Adipokinetic activity of the fractions were tested as described in the Materials and Methods (3.2.13.).Bars indicate means ± SEMs (6).

* $P<0.01$

** $P<0.05$

Figure II-1. Locust adipokinetic hormone-I - secondary structure demonstrating the postulated β-bend involving residues 5-8



(Redrawn from Mordue and Morgan, 1985)

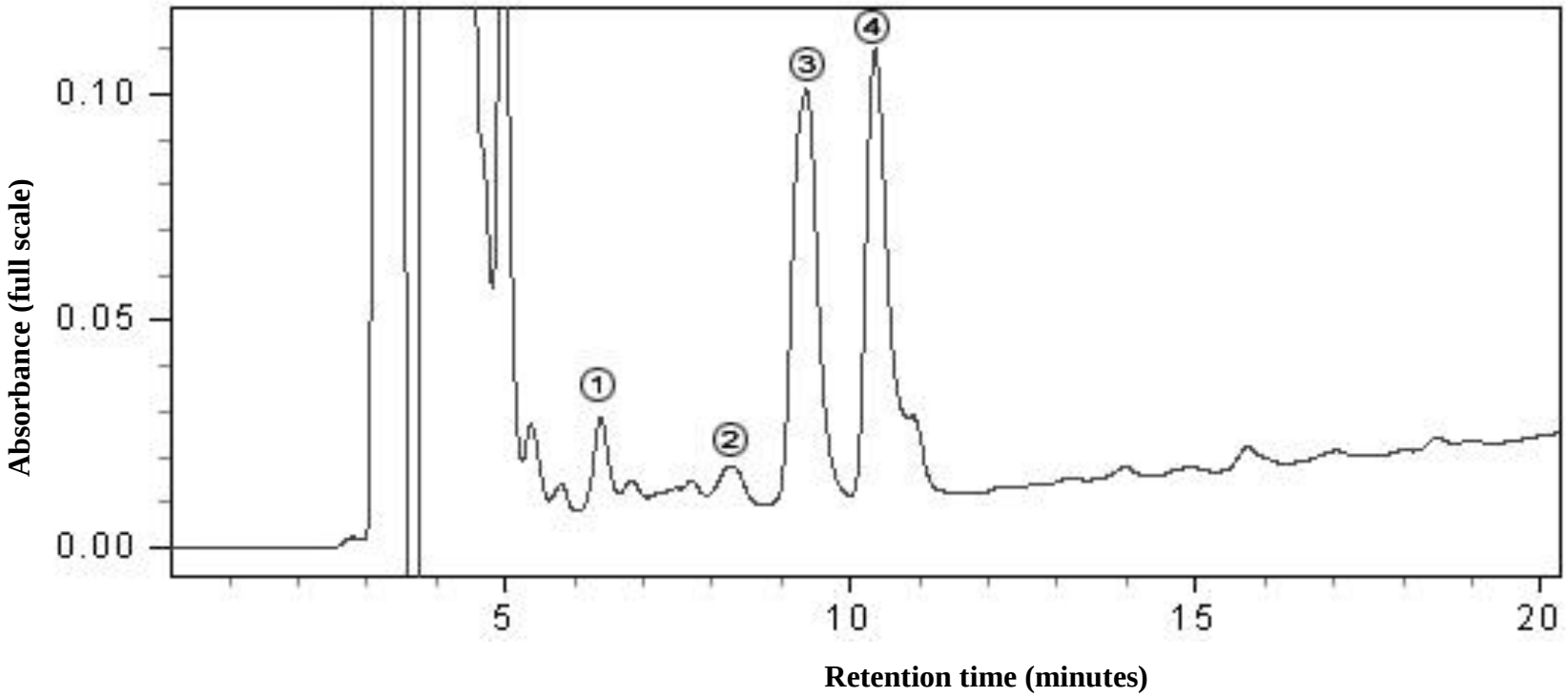


Figure IV-6. Elution profile of methanolic extract of brain-CC-CA complex of *I. limbata*. An extract made of 40 tissue complexes was fractionated using a C_{18} column with a binary gradient of 43% to 53% solvent B (60% acetonitrile in 0.05% aqueous TFA) against solvent A (0.05% aqueous TFA). The effluents were monitored at 210 nm.

Table II.1. Primary structures of peptides of the AKH/RPCH family

	Name	Amino acid sequence 1 2 3 4 5 6 7 8 9 10 11	Reference
1	*Vanessa* AKH	pGlu-Lys-Thr-Phe-Thr-Ser-Ser-Trp-Gly-Gly-Lys	Köllisch *et al*. (2000)
2	*Locusta* AKH-I	pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-ThrNH_2	Stone *et al*. (1976)
3	*Phymateus* AKH	pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-SerNH_2	Gäde *et al*. (1996)
4	*Decapotoma* CC	pGlu-Leu-Asn-Phe-Ser-Pro-Asn-Trp-Gly-AsnNH_2	Gäde (1995)
5	*Carausius* HrTH-I	pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-Gly-ThrNH_2	Gäde *et al*. (1992)
6	*Carausius* HrTH-II	pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-Gly-ThrNH_2	Gäde and Rinehart (1987 a)
7	*Phymateus* CC	pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-Gly-SerNH_2	Gäde and Kellner (1995)
8	*Tabanus* HOTH	pGlu-Leu-Thr-Phe-Thr-Pro-Gly-Trp-Gly-TyrNH_2	Jaffe *et al*. (1989)
9	*Heliothis* HrTH	pGlu-Leu-Thr-Phe-Ser-Ser-Gly-Trp-Gly-AsnNH_2	Jaffe *et al*. (1988)
10	*Romalea* CC	pGlu-Val-Asn-Phe-Thr-Pro-Asn-Trp-Gly-ThrNH_2	Gäde *et al*. (1988)
11	*Blaberus* HrTH	pGlu-Val-Asn-Phe-Ser-Pro-Gly-Trp-Gly-ThrNH_2	Hayes *et al*. (1986)
12	*Platypleura* HrTH I & II	pGlu-Val-Asn-Phe-Ser-Pro-Ser-Trp-Gly-AsnNH_2	Gäde and Janssens (1994)
13	*L. migratoria* HrTH	pGlu-Val-Thr-Phe-Ser-Arg-Asp-Trp-Ser-ProNH_2	Siegert (1999)

Contd …

	Name	Amino acid sequence 1 2 3 4 5 6 7 8 9	Reference
14	*Manduca* AKH	pGlu-Leu-Thr-Phe-Thr-Ser-Ser-Trp-GlyNH_2	Ziegler *et al*. (1985)
15	*Pseudagrion* AKH	pGlu-Val-Asn-Phe-Thr-Pro-Gly-TrpNH_2	Janssens *et al*. (1994)
16	*Libellula* AKH	pGlu-Val-Asn-Phe-Thr-Pro-Ser-TrpNH_2	Gäde (1990 c)
17	*Empusa* AKH	pGlu-Val-Asn-Phe-Thr-Pro-Asn-TrpNH_2	Gäde (1991 a)
18	Mantophasmatodea AKH	pGlu-Val-Asn-Phe-Ser-Pro-Gly-TrpNH_2	Gäde *et al*. (2005)
19	*Anax* AKH	pGlu-Val-Asn-Phe-Ser-Pro-Ser-TrpNH_2	Gäde *et al*. (1994 b)
20	*Periplaneta* CAH-I	pGlu-Val-Asn-Phe-Ser-Pro-Asn-TrpNH_2	Witten *et al*. (1984)
21	*Lethocerus* AKH	pGlu-Val-Asn-Phe-Ser-Pro-Tyr-TrpNH_2	Gäde *et al*. (2007 b)
22	*Gryllus* AKH	pGlu-Val-Asn-Phe-Ser-Thr-Gly-TrpNH_2	Gäde and Rinehart (1987 b)
23	*Tenebrio* HrTH	pGlu-Leu-Asn-Phe-Ser-Pro-Asn-TrpNH_2	Gäde and Rosinski (1990)
24	*Corixa* AKH	pGlu-Leu-Asn-Phe-Ser-Pro-Ser-TrpNH_2	Gäde *et al*. (2007 a)
25	*Pandalus* RPCH	pGlu-Leu-Asn-Phe-Ser-Pro-Gly-TrpNH_2	Fernlund and Josefsson (1972)
26	*Locusta* AKH-II	pGlu-Leu-Asn-Phe-Ser-Ala-Gly-TrpNH_2	Siegert *et al*. (1985)
27	*Schistocerca* AKH-II	pGlu-Leu-Asn-Phe-Ser-Thr-Gly-TrpNH_2	Siegert *et al*. (1985)

Contd …

	Name	Amino acid sequence 1 2 3 4 5 6 7 8	Reference
28	*Nepa* AKH	pGlu-Leu-Asn-Phe-Ser-Ser-Gly-TrpNH_2	Gäde *et al*. (2007 b)
29	*Melolontha* CC	pGlu-Leu-Asn-Tyr-Ser-Pro-Asp-TrpNH_2	Gäde (1991 b)
30	*Onitis* CC-I	pGlu-Tyr-Asn-Phe-Ser-Thr-Gly-TrpNH_2	Gäde (1997)
31	*Onitis* CC-II	pGlu-Phe-Asn-Tyr-Ser-Pro-Asp-TrpNH_2	Gäde (1997)
32	*Locusta* AKH-III	pGlu-Leu-Asn-Phe-Thr-Pro-Trp-TrpNH_2	Oudejans *et al*. (1991)
33	*Pyrrhocoris* AKH	pGlu-Leu-Asn-Phe-Thr-Pro-Asn-TrpNH_2	Kodrik *et al*. (2000)
34	*Microhodotermes* CC	pGlu-Ile-Asn-Phe-Thr-Pro-Asn-TrpNH_2	Liebrich *et al*. (1995)
35	*Phymateus* AKH-III	pGlu-Ile-Asn-Phe-Thr-Pro-Trp-TrpNH_2	Siegert *et al*. (2000)
36	*Polyphaga* HrTH	pGlu-Ile-Thr-Phe-Thr-Pro-Asn-TrpNH_2	Gaede and Kellner (1992)
37	*Periplaneta* CAH-II	pGlu-Leu-Thr-Phe-Thr-Pro-Asn-TrpNH_2	Witten *et al*. (1984)
38	*Tabanus* AKH	pGlu-Leu-Thr-Phe-Thr-Pro-Gly-TrpNH_2	Jaffe *et al*. (1989)
39	*Phormia* HrTH	pGlu-Leu-Thr-Phe-Ser-Pro-Asp-TrpNH_2	Gäde *et al*. (1990)
40	*Erythemis* AKH	pGlu-Lys-Asn-Phe-Thr-Pro-Ser-TrpNH_2	Gäde and Kellner (1999)