MECHANISM OF ACTIVATION OF LIPOLYSIS IN *MANDUCA SEXTA*: ROLE OF TRIGLYCERIDE-LIPASE

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ABBREVIATIONS

A-kinase	cAMP dependent protein kinase A
AKH	Adipokinetic Hormone
ADRP	Adipose differentiation-related protein
ATP	Adenosine triphosphate
BEL	Bromoenol-lactone
cAMP	Cyclic adenosine monophosphate
DG	Diacylglycerol
DEAE	Diethylaminoethyl cellulose
DEDA	7,7-dimethyleicosadienoic acid
DTT	dithiothretiol
EDTA	Ethylenediamietetraacetic acid
FFA	Free fatty acid
HDLp	High density lipophorin
HSL	Hormone sensitive lipase
^{3}H	Tritium
LDLp	Low density lipophorin
Lp	Lipophorin
LTP	Lipd transfer protein
LD	Lipid droplets
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine

Lsdp1	Lipid storage droplet protein 1
MAFP	Methyl arachinodyl fluorophosphonate
MALDI-TOF	Matrix assisted laser desorption ionization time of flight
MOPS	3-(N-morpholino) propane-sulfonic acid
MG	Monoglyceride
MS	Mass spectrometry
PA	Phospholipase
PAGE	Polyacrylamide gel electrophoresis
PA-PLA_1	Phosphatidic acid preferring-phospholipase A_1
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
Peri A	Perilipin A
PKA	cAMP dependent protein Kinase A
PKC	Protein kinase C
PL	Phospholipids
PLA_1	Phospholipase A_1
PLA_2	Phospholipase A_2
TG	Triglycerides
TIP47	Tail interacting 47-kDa protein
TLC	Thin layer chromatography

CHAPTER- I

INTRODUCTION

Insects are the most abundant form of animal life on the planet. One of the most important characteristics of insects is their ability to fly. The fossil record indicates that flying insects evolved at least 300 million years ago (Dalton 1977, Wootton 1986). Humans often consider insects as pests and as disease spreading bugs. In fact, life as we know today would not be possible without insects. They play very important role in plant pollination and play a critical role in removing dead material from the biosphere-without insects we would soon be buried in debris.

Flight is a form of locomotion that is dependent on a high rate of energy metabolism. The metabolic rate of flying insects can be 20-100 times that of resting animals and are among the highest known in nature (Beenakkers et al., 1984; Kammer and Heinrich, 1978). This massive metabolic rate of flying insects provides an attractive model system to study regulation of metabolism during physiological exercise. Insects and vertebrates share many common metabolic pathways, thus are useful models that can assist our general understanding of biology.

Flight fuels

Insects have been classified broadly in different categories according to their usage of substrates as fuel for flight. Insects use carbohydrate, lipids, amino acids or a combination of two or more of these as flight fuel. Each fuel has advantages and disadvantages and insects have adopted particular substrates or combinations of substrates for optimum fuel selection in order to suit particular physiological requirements.

Carbohydrates are a sole source of energy for most of the short distance flying insects including Diptera, Hymenoptera and some Lepidoptera. Carbohydrate is stored in the form of glycogen in flight muscles, fat body and gut, but the major carbohydrate pool in many insects is hemolymph trehalose (Rankin and Burchsted, 1992). Use of carbohydrates for flight is advantageous in some contexts because it is highly soluble in aqueous medium and easily available for immediate metabolism. The primary disadvantage of carbohydrates as a flight fuel source is their bulky nature i.e hydration of stored glycogen makes it approximately eight times heavier than isocaloric amounts of fat (Weis-Fogy, 1952).

The amino acid proline is used as a primary flight fuel in some insects. The dipteran tsetse fly, *Glossina morsitans* and Colorado potato beetle, *Leptinotarsa decemlineata* use partial oxidation of the amino acid, proline, as fuel for flight (Weeda, 1980; Bursell, 1981, Gade and Auerswald, 2002). Alanine and triglycerides (TG) are the major renewable source of proline synthesis from fat body (Weeda et al., 1979). The physiological advantage of using proline rather

than diacylglycerols is that proline is water-soluble and avoids the metabolic expense of lipoprotein carrier mechanisms (Wheeler 1989). However, the stored fuel utilized for long distance flight is stored lipids.

Most of the long-distance flying insects, including *Manduca sexta* and locust, mobilize both carbohydrates and lipid reserves as fuels for flight. The initial short period of flight is powered mainly by carbohydrate; the flight fuel then switches to lipids for sustained flight (Weis-Fogh, 1952; Beenakkers et al., 1984). The majority of lipid is stored in masses and sheets of adipose tissue, collectively called the fat body (Law and Wells, 1989). The lipids are stored primarily as triacylglycerols, which comprise >90% of total fat body lipids (Bailey, 1975). According to Brusell (1963) the yield of energy per unit mass of material oxidized is 0.18 mol ATP/g for glucose and 0.52 for proline as compared with values of 0.65 mol ATP/g of fat. In addition to high energy value, for weight economic reasons (since lipids do not require water for their storage), lipid appears to be by far the more desirable substrate for insect flight energetics.

Fundamental endocrine regulation of energy homeostasis is observed from lower invertebrates to higher organisms. For example, bomboyxin, insulin-related peptide of insects, reduces hemolymph sugar concentrations in a dose-dependent manner in the silkworm *Bombyx mori* (Satake, et al., 1997). In addition, genes encoding Drosophila insulin-like peptides (*dilp*) have also been identified (Brogiolo et al. 2001), and transgenic ablation of *dilp*-producing neurons results in the elevation of total blood sugar (Rulifson et al. 2002). The most studied insect hormone to play a central role in energy metabolism is a functional

homolog of mammalian glucagon, adipokinetic hormone (AKH). AKH was first discovered as a lipid mobilizing hormone in migratory locusts (Beenakers, 1969; Mayers and Candy, 1969). AKHs are a large family of 8-10 amino-acids peptides secreted into hemolymph by the neurosecretory cells of the corpora cardiaca (Orchard, 1987; Arrese et al., 1996). These peptide hormones form the largest neuropeptide family in arthropods, including >30 isoforms identified in >80 species encompassing all major insect phyla and several crustacean species (Gade et al., 1997). Since its discovery, several studies have shown that AKH not only mobilizes lipids (Arrese et al., 1997; Gade et al., 1997), but also mobilizes stored carbohydrate (Gade et al., 1997; Van der horst et al. 2001) and causes hyperporlinaemia in the hemolymph of beetle species (Gade and Auerswald, 2002). Injection of AKH in adult *M. sexta* stimulates mobilization of stored TG by activating TG-lipase (Ziegler et al., 1990; Arrese et al, 1996), whereas in the larval stages the same hormone induces the mobilization of glycogen by activating glycogen phosphorylase (Ziegler et al., 1990). Other studies have reported similar effect of AKH in cockroaches (Bedford 1977), locusts (Gade et al., 1997) and fruit fly, *D. melanogaster* (Lee and Park 2004). In some insects, AKH has also been shown to mobilize one energy reserve and not another. For example, in the horse fly (*Tabanus atratus*), injection of AKH causes hyperlipemia but not hypertrehalosemia (Jaffe et al. 1989) and in the blow fly (*Phormia terraenovae*), it causes hypertrehalosemia and not hyperlipemia (Gade et al. 1990).

Since lipid is a major fuel for flight for most of the long distance flying insects, it is very important to understand lipid metabolism for; 1) advance knowledge of insect physiology, 2) economical reasons, since insects are pest to many crops, consume agriculture products and transmit diseases, 3) in many ways, fat metabolism in insects is less complex than in vertebrates, so it could serve as a simple model system to advance fundamental aspects of fat metabolism. Most of the current information about lipid metabolism in insects comes from studies carried out in *L. migratoria* and *M. sexta*. To advance the understanding of lipid metabolism in insects, in this study, we are using *M. sexta* as a model organism.

***Manduca sexta* life cycle**:

The tobacco hornworm, *M. sexta,* is a moth with three different life stages, the larva, pupa, and adult (**Figure 1**). During the larval period, ~20 days, insect feeds constantly, and the content of fat body increases continuously until the end of larval development. During the larval stage, the content of TG in the fat body increases from a few µg to ~80 mg (Fernando-Warnakulasuriya et al., 1988). During subsequent development, the lipid reserves are used to sustain the life of the adult insect, which feeds occasionally (Fernando-Warnakulasuriya et al., 1988; Ziegler, R., 1991; Arrese et al., 2001; Canavosa et al., 2001). Due to these metabolic features, *M. sexta* represents an excellent model for studying the basic mechanisms involved in the mobilization of TG in adult insects (moth).

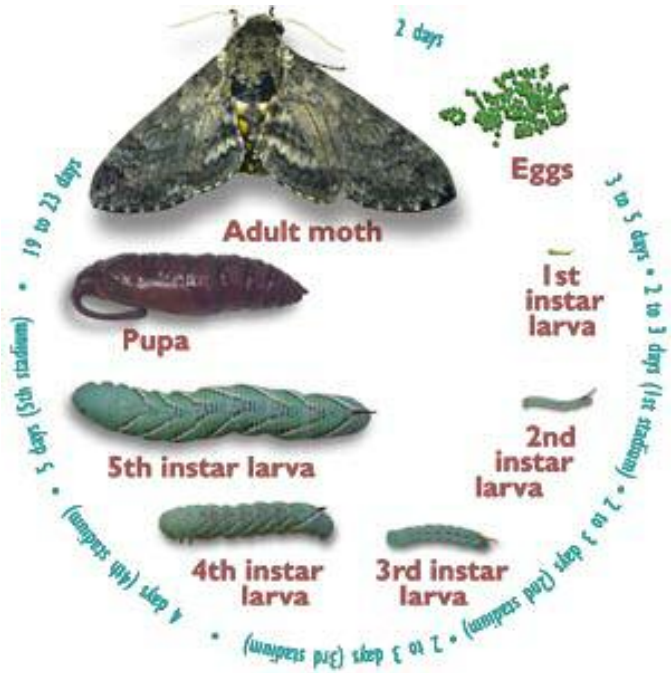


Figure 1: *Manduca sexta* life cycle
(**Source**: http://insected.arizona.edu/manduca/Mand_cycle.html)

Overview of lipid mobilization in *M. sexta* during flight activity

Triacylglycerol (TG) is stored in fat body adipocytes as cytosolic lipid droplets (Willott et al., 1988). The mobilization of stored TG is induced by two kinds of hormones: adipokinetic hormone (AKH) (Beenakkers, et al., 1985) and octapamine (Orchard et al., 1982; Fields and Woodring, 1991). Unlike vertebrates in which stored lipids are mobilized as free fatty acids (FFA), insects mobilize lipids in the form of diacylglycerol (DG) (Chino and Gilbert, 1964; Beenakkers et al., 1985; Arrese et al., 1997). During energy demanding processes like flight and reproduction, AKH is secreted into hemolymph and exerts its effects on lipid mobilization via signal transduction (**Figure 2**).

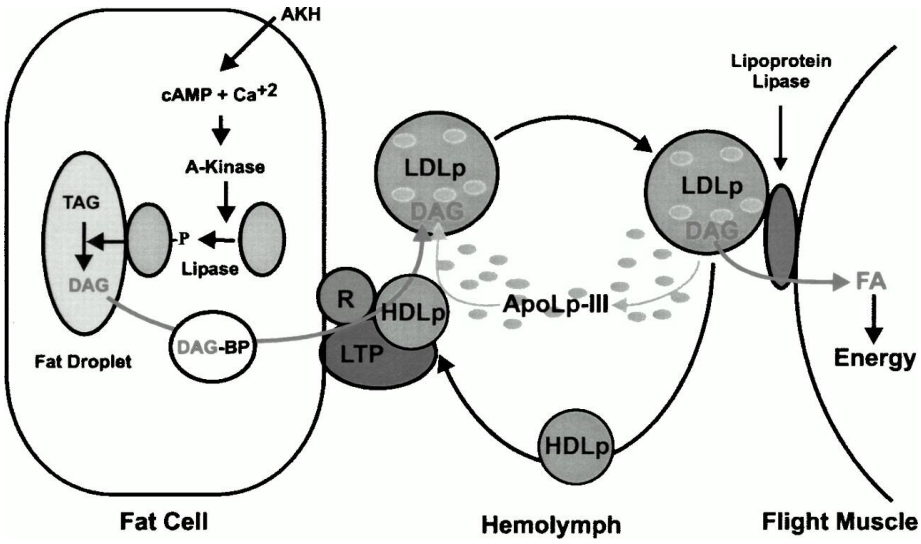


Figure 2. Schematic overview of lipid mobilization and transport in *M. sexta* during flight activity: Adipokinetic hormone (AKH) stimulates diacylglycerol (DAG) production and secretion from the fat body. DAG is produced by the action of a lipase acting on the stored triacylglycerol (TAG) and is transported to the plasma membrane via a DAG-binding protein (DAG-BP). Once in the membrane, the DAG leaves the cell and is added to high-density lipophorin (HDLp) with the assistance of lipid transfer particle (LTP) to produce LDLp. LDLp is stabilized by binding apolipophorin-III (apoLp-III). LDLp moves to the muscle cell where the DAG is hydrolyzed by a lipophorin lipase. After delipidation, apoLp-III dissociates and LDLp is converted back to HDLp. HDLp then cycles back to the fat body to pick additional DAG and apoLp-III. FA, Fatty acid; R, receptor. (**Source**: Canavosa et al., 2001)

The sequence of events leading to the stimulation of lipolysis induced by AKH is still not clear (Gade and Auerswald, 2003). Binding of AKH to its receptor (Ziegler et al., 1995) causes a sustained increase in intracellular calcium influx and activation of adenylate cycalse, giving rise to two intracellular messengers, calcium and cAMP (Gade and Holwerda, 1976; Lum and Chino, 1990; Arrese et al., 1999; Van der Horst et al., 1999). AKH has also been shown to increase intracellular concentration of inositol (1, 4, 5)-triphosphate in two locusts, *Schistocerca gregaria* (Stagg and Candy, 1996) and *L. migratoria* (Van Marrewijk et al., 1996; Vroemen et al., 1998). Injection of AKH in adult *M. sexta* also

increases intracellular cAMP dependent protein kinase A (PKA) activity, which confirms intracellular increase and involvement of cAMP in lipid metabolism (Arrese et al., 1999). In addition to increase in PKA activity, in adult *M. sexta*, AKH activates fat body TG-lipase and this activation precedes the appearance of DG in hemolymph (Arrese et al., 1996b). AKH also has been shown to activate fat body TG-lipase in locust *Schistocerca gregaria* (Ogoyi et al., 1998). Beyond activation of TG-lipase, other roles of these intracellular messages have not been characterized. Once DG is synthesized, it is exported out of the fat body cell by an unknown mechanism, and loaded into high density lipophorin (HDLp), which requires lipid transfer particle (LTP) (Van Heusden and Law, 1989). This causes transformation of HDLp into low density lipophorin (LDLp), which transports DG to the sites of utilization, *e.g*. the flight muscle, and ovaries, where it is hydrolyzed to free fatty acids by a lipoprotein-lipase (Soulages and Wells, 1994).

Because the PKA activation by AKH precedes the activation of the TG-lipase, which in turn precedes the appearance of DG in circulation, the stimulation of lipolysis is presumably regulated by phosphorylation reactions. Given the role of PKA in AKH-induced activation of the lipolysis, in this study, we are investigating the role of PKA and the role of proteins targeted by PKA in the activation of lipolysis in the insect fat body using *in vivo* and *in vitro* experiments.

CHAPTER-II

cAMP-DEPENDENT PROTEIN KINASE OF MANDUCA SEXTA PHOSPHORYLATES BUT DOES NOT ACTIVATE THE FAT BODY TRIGLYCERIDE LIPASE

INTRODUCTION

Fatty acids are the primary substrate used by insects to fuel long-term flight. Fatty acids are stored as triacylglycerol (TG) and the vast majority of TG stores are in the fat body (Bailey, 1975; Canavoso et al., 2001) in the form of lipid droplets (Willott et al., 1988). In the tobacco hornworm, *Manduca sexta*, the maximum content of fat body TG occurs at the end of larval development, as a consequence of the accumulation of reserves during larval feeding (Fernando-Warnakulasuriya et al., 1988). Afterwards, during the subsequent non-feeding pupal and adult periods, the TG stores decline (Ziegler, 1991).

Utilization of the fatty acids stored in the fat body requires hydrolysis of TG in a reaction catalyzed by a TG-lipase. Unlike vertebrates, in which stored fatty acids are mobilized as free fatty acids (FFA), a great number of insects mobilize fatty acids as diacylglycerol (DG) (Beenakkers et al., 1985). Lipolysis is regulated by adipokinetic hormone (AKH) (Orchard, 1987; Van der Horst, 2003). The sequence of events leading to the stimulation of lipolysis induced by AKH still is not well understood (Gäde and Auerswald, 2003). The AKH receptors from the fruit fly *Drosophila melanogaster* and the silkworm *Bombyx mori* have been

recently identified (Hauser et al., 1997; Staubli et al., 2002). These receptors are related to the mammalian gonadotropin releasing hormone receptors (GnRHR), which is a G protein-coupled receptor that activates both inositol phosphate and cAMP signaling responses. This includes the activation of phospholipase C, adenylyl cyclase, and ion channels that regulate the intracellular levels of inositol phosphate, calcium, cAMP, and other second messengers (Arora et al., 1998). The present data supports a model in which binding of AKH to its receptor leads to a Gs-coupled activation of adenylyl cyclase. Besides the involvement of cAMP, the lipolytic response of AKH in *M. sexta* also induces a sustained increase in calcium influx but the processes mediated by Ca^{+2} remain unknown (Arrese et al., 1999). The influx of extracellular Ca^{+2} is also essential for lipid release from fat body of locust, *Locusta migratory* (Lum and Chino, 1990; Wang et al., 1990).

Fat body TG-lipase catalyzes the hydrolysis of TG and it is expected to play a central role in the regulation of lipolysis in *M. sexta* (Arrese et al., 1996a; Arrese et al., 1997). The TG-lipase that has been purified from the fat body of adult *M. sexta* (Arrese and Wells, 1994) is the only purified lipase from insects. The enzyme is a single polypeptide of 76 kDa that has several properties in common with the vertebrate hormone-sensitive lipase (HSL), which catalyzes the rate-limiting step in mobilization of adipose tissue fatty acids (Kraemer and Shen, 2002). In adult *M. sexta*, AKH activates the fat body TG-lipase and this activation precedes the appearance of DG in the hemolymph (Arrese et al., 1996b). Likewise, AKH rapidly activates cAMP-dependent protein kinase from the fat body of *M. sexta* (Arrese et al., 1999). Because the kinase activation precedes

the activation of the lipase, which in turn precedes the appearance of DG in circulation, the stimulation of lipolysis induced by AKH is presumably regulated by phosphorylation reactions. In this context, cAMP-dependent protein kinase emerges as a central player of the transduction of the stimulus that controls the mobilization of stored lipids in the fat body.

AKH stimulation of the lipolysis promotes a rapid two-fold increase in the content of hemolymph lipids. DG comprises 95% of the total hemolymph lipids (Arrese and Wells, 1997) in adult *M. sexta.* Concomitant to the increase in hemolymph lipids, stimulation of lipolysis promotes an increase in the DG content of lipid droplets and the cytosolic fraction (Arrese et al., 2001).

Given the apparent role of cAMP in AKH-induced activation of the lipolysis, we are investigating the role of PKA and the reversible phosphorylation of the TG-lipase on the lipolytic activity of the insect fat body. In order to investigate this issue, the catalytic subunit of the cAMP-dependent kinase was purified from the fat body of adult *M. sexta* insects. The properties of PKA and its role on the direct activation of purified lipase are described here.

EXPERIMENTAL PROCEDURES

Insects: *M. sexta* eggs were purchased from Carolina Biological Supplies (NC), and larvae were reared on artificial diet (Bell and Joachim, 1976). Adults were kept at room temperature without food. Two- or three-day-old adults *M. sexta* were used as experimental insects**.**

Materials: [γ-^{32}P]ATP was purchased from ICN Biomedical (Irvine, CA). cAMP, leupeptin, aprotonin, Triton X-100, benzamidine, bovine PKA catalytic subunit, histone II AS, all peptide substrates and inhibitors were obtained from Sigma (St Louis, MO). DEAE-cellulose (DE-52) and P81 phosphocellulose filter paper were purchased from Whatman (Hillsboro, OR). Q-Sepharose was purchased from Amersham Pharmacia (Piscataway, NJ). Anti-human catalytic subunit of PKA antibody was obtained from Upstate Biotechnology (Lake Placid, NY). All other chemicals were of analytical grades.

Measurement of A-kinase activity: A-kinase activity was measured as described previously (Arrese et al., 1999). The final assay volume of 0.1 ml contained 50 mM MOPS (3-(N-morpholino)propane-sulfonic acid), pH 7.0, 10 mM magnesium acetate, 0.5 mM EDTA (ethylenediaminetetraacetic acid), 600 µM histone II AS, 0.2 mM [γ-^{32}P]ATP (1.5×10^6 cpm/nmole) and 10 µM cAMP when required. After incubation at room temperature for 15 min, the reaction was terminated by the addition of 5 µl of 6 N HCl. Seventy µl of the reaction mixture was spotted onto a disc of phosphocellulose filter paper (2.5 cm), and the filters were washed for 20 min in 50 mM NaCl four times (Roskoski, 1983). The radioactivity associated with the dried filters was counted by liquid scintillation counter using a Packard Tricarb 1900 TR. Under these conditions the kinase activity was linear up to the addition of 0.5 mg of total protein in the incubation mixture. Kinase activity was expressed in nanomol of phosphate transferred to histone per minute.

When synthetic peptide was used as phosphate acceptor for the purified kinase, the reaction mixture contained 50 µM peptide substrate, 50 mM MOPS, pH 7.0, 0.5 mM magnesium acetate, 0.25 mM EDTA, and 0.2 mM [γ-^{32}P]ATP (0.5×10^5 cpm/nmole).

Purification of M. sexta PKA catalytic subunit: All steps were carried out on ice or at 4 °C. Fat body tissue from 100 insects was collected in homogenizing buffer (20 mM Tris, pH 7.4, 0.25 M sucrose, 1 mM EDTA, 0.1 mM benzamidine, 10 mg/l leupeptine, 1 mg/l aprotonin and 0.1% (v/v) 2-mercaptoethanol). The tissue was homogenized at a ratio of 3 ml per fat body using a Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was subjected to ultracentrifugation at 100,000×*g* for 1 h and the soluble extract was collected. The soluble extract was loaded onto a DE-52 column (3×10 cm) equilibrated with buffer A (50 mM Tris-HCl, pH-7.4, 1 mM EDTA, 0.1% 2-mercaptoethanol). The column was washed with 5 volumes of buffer A and developed with a linear salt gradient from 0–400 mM NaCl in buffer A. Fractions of 8 ml were collected and those containing PKA activity (eluted between 110–200 mM NaCl) were pooled, dialyzed against buffer A containing 30 mM NaCl and subjected to a second DE-52 column (2×5 cm) equilibrated with buffer A containing 30 mM NaCl. After extensive wash of the column with equilibration buffer, a solution of 1 mM cAMP in buffer A was passed through the column. Fractions containing kinase activity were pooled and the concentration of NaCl was increased to 50 mM and loaded onto a Q-sepharose anion exchanger column (2×1 cm) equilibrated with buffer A containing 50 mM NaCl. Under this condition the majority of kinase activity did

not bind to the resin and was found in the flow trough. These fractions were collected, pooled and stored on ice until further use. For prolonged storage, the enzyme activity was preserved for several months at −20 °C in the presence of 50% glycerol.

Other methods: Protein concentrations were determined by the Bradford dye-binding assay (Bradford, 1976) using bovine serum albumin as standard. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970) and proteins were visualized by Coomassie Brilliant Blue R staining.

For Western analysis, proteins were transferred to nitrocellulose membrane in a Hoeffer transfer unit (Amersham Pharmacia). Immunoblot analysis was performed using 1 µg/ml of anti-catalytic subunit of human PKA (rabbit polyclonal). The positive signal recognized by anti-PKAc was detected using HRP-conjugated anti-rabbit secondary antibody (1:5000). Immunoreactive protein was visualized by autoradiography using chemiluminescence (Chemilucent detection system from Chemicon, Temecula, CA).

For the Peptide Mass Fingerprinting, purified PKA catalytic subunit was separated on 10% SDS-PAGE and the band that was visualized by Coomassie staining was excised, minced and destained using 100% acetonitrile, followed by four washes in 1 ml water. The gel pieces were incubated for 20 min in 500 µl of 100 mM ammonium bicarbonate followed by 20 min incubation with 500 µl of 50% acetonitrile in 50 mM ammonium bicarbonate. Gel pieces were dried under

vacuum, rehydrated and digested with 50 ng/µl trypsin (sequencing grade, Promega, Madison, WI) in 25 mM ammonium bicarbonate at 4 °C overnight. Peptides were extracted and analyzed by MALDI-TOF mass spectrometry at the Department of Biochemistry, Oklahoma State University. Mass characterizations were performed using *α*-cyano-4-hydroxycinnamic acid as matrix, using external standards as calibrants. MSDB database was used to identify the peptides shown in **Figure 5**.

Phosphorylation of TG-lipase by PKAc: Fat body TG-lipase was purified as described previously (Arrese and Wells, 1994). TG-lipase (7 µg) was incubated in a final volume of 0.1 ml containing 50 mM MOPS, 1 mM magnesium acetate, 0.5 mM EDTA, 1 mM dithiothretiol, purified PKAc (0.25 units) and 0.2 mM [γ-^{32}P]ATP (5×10^6 cpm/nmol). After 15 min incubation at room temperature, the reaction was stopped by addition of electrophoresis sample buffer and the sample was analyzed by SDS-PAGE. Dried gels were then exposed to X-ray film. Control incubations in which purified PKAc was omitted indicated the absence of PKA activity, or other kinase activators, in the preparation of TG-lipase. On the other hand, when purified PKAc was incubated with [γ-^{32}P]ATP neither phosphorylation of PKAc (autophosphorylation) nor any other phosphorylation of endogenous substrates was observed. Unlabeled phosphorylated TG-lipase was made using the same conditions, except unlabeled ATP was used and the reaction was stopped by addition of 5 mM EDTA. Lipase activity of the phosphorylated and non-phosphorylated (control)

form of the enzyme was measured using micellar [^{3}H]-triolein as described previously (Arrese and Wells, 1994).

Dephosphorylation and rephosphorylation of TG-lipase: A similar experiment was conducted using dephosphorylated TG-lipase. For this purpose, purified enzyme (7 µg) was first incubated with ten units of Alkaline Phosphatase Type VIII from Sigma in a buffer containing 20 mM Tris pH 8.8 and 1 mM $MgCl_2$ for 30 min at room temperature. Then Phosphatase Inhibitor Cocktail I from Sigma was added (1:100 dilution) and the samples were dialyzed against the PKAc reaction buffer (50 mM MOPS, 1 mM magnesium acetate, 0.5 mM EDTA, 1 mM DTT). The lipase was phosphorylated as indicated above, analyzed by SDS-PAGE followed by autoradiography, and the effect of phosphorylation on lipase activity was determined.

Statistics: Results are presented as the mean±SEM. Statistical comparisons were made by *t* test and $p \leq 0.05$ was considered to be significant.

RESULTS AND DISCUSSION

Purification of M. sexta PKA catalytic subunit

The occurrence of PKA activity in adult *M. sexta* fat body was studied in tissue homogenate. The kinase activity of homogenates increased 450 ± 33 % in response to stimulation by cAMP suggesting a high PKA activity. Moreover, this increase was abolished by the specific inhibitor of PKA, PKI. Previous studies have shown PKA activity in *Locusta migratoria* fat body (Pines and Applebaum, 1978) and several other insect tissues (Bodnaryk, 1983; Baghdassarian-Chalaye et al, 1988; Bishoff et al.,1990; Cho et al 1999).

cAMP-dependent protein kinase (PKA) is composed of two catalytic subunits and two regulatory subunits. The holoenzyme is inactive. Upon cAMP binding, the enzyme activates by releasing the catalytic subunits (Taylor et al, 2004). The large difference between the isoelectric points of the free catalytic (basic) and the regulatory subunit (acidic) has been used to facilitate the purification of the catalytic subunit from vertebrate tissues (Reimann and Beham, 1983). Using differential binding capacity on anion exchanger resin we developed a procedure for the purification of the catalytic subunit of PKA from the insect fat body (**Table 1**). Preliminary studies indicated that the vast majority of PKA is associated with the cytosolic fraction which is obtained as the soluble material after centrifugation (100,000xg) of fat body homogenate and removal of the fat cake. This material was subjected to DE-52 column chromatography. A linear concentration gradient of NaCl was used to elute the proteins from the column. Kinase activity inducible by cAMP was monitored in individual fractions by

measuring the activity in the presence and absence of cAMP. Up to 400% increase of kinase activity induced by cAMP was found in fractions 16 to 35 (110-220 mM NaCl) indicating that PKA holoenzyme was eluting in this region as a single peak (**Figure 3**). These fractions were pooled, dialyzed and subjected to a second DE-52 chromatography. After extensive wash, the catalytic subunit of PKA was dissociated and eluted from the column using 1mM cAMP.

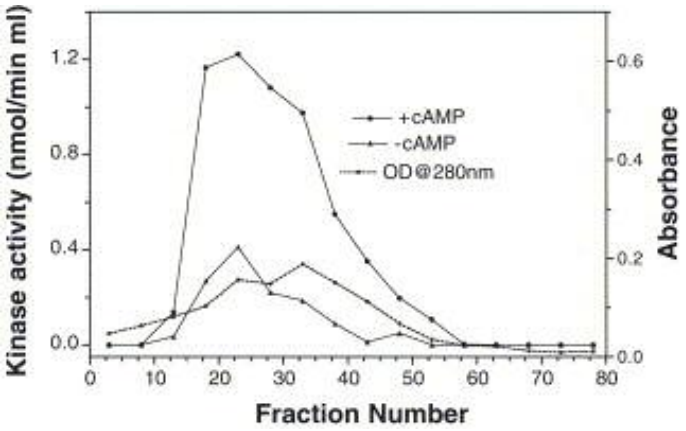


Figure 3. Elution profile of PKA from DE-52(I) column. The soluble extract after 100,000x g centrifugation of adult M.sexta fat body homogenate was loaded into the column. The column was developed with 0-400 mM linear sodium chloride gradient in buffer A. Aliquots from fractions were assayed for A-kinase activity in presence (—•—) and absence (—Δ—) of 10 μM cAMP. Absorbance was monitored at 280 nm (--0--) of individual fractions.

The kinase activity eluted as a very sharp peak but was not pure (Figure 4, lane 2). To remove the contaminants a strong anion exchanger, Q-Sepharose, was used. This was based on the observation that proteins co-eluting with PKA in DE-52 chromatography bind to the Q-Sepharose resin equilibrated in buffer containing 50 mM NaCl, while catalytic subunit of PKA does not bind. The majority (75%) of kinase activity was found in the flow through having a specific activity of 266 nmoles / mg-min (**Table 1**).

Table 1.Purification of the catalytic subunit of PKA from *M. sexta* fat body

	Total protein (mg)	**Specific activity (nmol/mg-min)**	**Recovery (%)**	**Purification (fold)**
Cytosolic fraction	*1662*	*0.15*[a]	*100*	1
DE-52 (I)	*226*	*0.78*[a]	*71*	5
DE-52 (II)	*1.60*	*54*	*35*	360
Q-Sepharose	0.12	266	13	1773

[a] The enzyme activity was assayed in the presence of 10 μM cAMP.

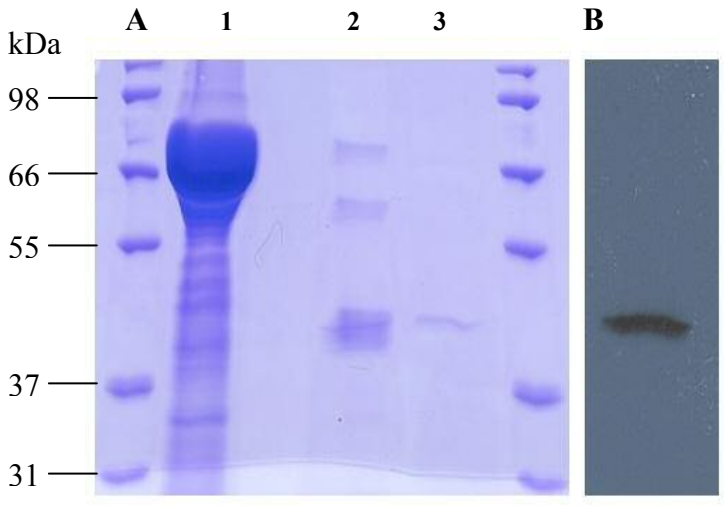
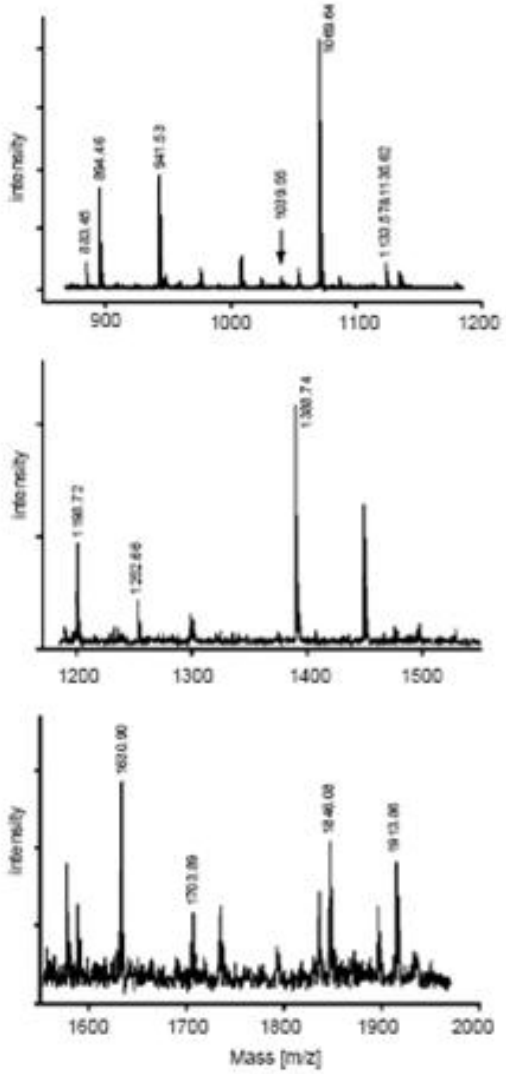


Figure 4. SDS-PAGE and Western blot analysis of purified PKA**.** A) Proteins were resolved in 10%-acrylamide gel and stained with Coomassie Brilliant Blue. Lanes: (1) soluble extract; (2) after DE-52 (II); (3) after Q-Sepharose. B) Western blot analysis of final preparation using human anti-catalytic subunit

SDS-PAGE analysis showed that this preparation contained a single protein band with an apparent molecular mass of 45.1 ± 0.2 kDa (**Figure 4A**, lane 3). Similar sizes have been reported for the catalytic subunits of PKA from *Drosophila melanogaster* (Foster et al., 1984; Haracksa and Udvardy, 1992), the gall insects *Epiblema scudderiana* and *Eurosta solidaginis* (Pfister and Storey,

2002), and other animals (Taylor et al., 1990; Mehrani and Storey, 1995; Brooks and Storey, 1996). Moreover, Western blot analysis of the final preparation using polyclonal antibodies against human catalytic subunit of PKA recognized this protein band (**Figure 4B**).



Mass	Predicted peptides
1703.89	NPTNTAALDDFERIK
1135.63	IKTLGTGSFGR
894.46	TLGTGSFGR
1252.67	QVEHTLNEKR
1845.08	DLKPENLLIDSQGYLK
883.45	VTDFGFAK
1039.56	VTDFGFAKR
1388.75	VRFPSHFGSDLK
1133.58	FPSHFGSDLK
1650.91	FPSHFGSDLKDLLR
1198.72	NLLQVDLTKR
1069.64	KIEAPFIPR
941.54	IEAPFIPR
1913.86	GPGDTSNFDDYEEEALR

Figure 5. MALDI-TOF analysis of purified kinase after cleavage by trypsin. Table shows the predicted sequence of the peptides that matched to several regions along the sequence of *Drosophila melanogaster* PKA, C31751.

The identity of the protein was confirmed further by trypsin fragmentation followed by MALDI-TOF analysis (**Figure 5**)

The search for matching peptides identified 14 peptides that coincided precisely to the *Drosophila melanogaster* catalytic subunit of cAMP-dependent protein kinase (C31751) (**Figure 6**). The correlation of the match was significant and the peptide matches covered 31% of the drosophila sequence. Further NCBI Blast search of individual peptides gave 97% identity to *Anopheles gambiae* and *Apis mellifera* and 88% to human catalytic subunit of PKA. This similarity is in good agreement with the conclusion of other authors that the catalytic subunit of PKA is highly conserved from lower to higher eukaryotes (Foster et al., 1984; Taylor et al., 1990; Denis et al., 1991; Haq et al., 2000; Pfister and Storey, 2002).

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  1 MGNNATTSNKKVDAAETVKEFLEQAKEEFEDKWRRNPTNTAALDDFERIKTLGTGSFGRV
 61 MIVQHKPTKDYYAMKILDKQKVVKLKQVEHTLNEKRILQAIQFPFLVSLRYHFKDNSNLY
121 MVLEYVPGGEMFSHLRKVGRFSEPHSRFYAAQIVLAFEYLHYLDLIYRDLKPENLLIDSQ
181 GYLKVTDFGFAKRVKGRTWTLCGTPEYLAPEIILSKGYNKAVDWWALGVLVYEMAAGYPP
241 FFADQPIQIYEKIVSGKVRFPSHFGSDLKDLLRNLLQVDLTKRYGNLKAGVNDIKNQKWF
301 ASTDWIAIFQKKIEAPFIPRCKGPGDTSNFDDYEEEALRISSTEKCAKEFAEF
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Figure 6. Aminoacid sequence of cAMP-dependent catalytic chain of *Drosophila melanogaster* (C31751). The matched peptides from *M. sexta* are shown underlined.

Characterization of M. sexta PKA catalytic subunit

The ability of purified enzyme to phosphorylate Kemptide was investigated. This is a synthetic heptapeptide corresponding to a part of the phosphorylation site of porcine liver pyruvate kinase that is an excellent substrate for PKA (Kemp et al., 1977; Foster et al., 1984; Denis et al., 1991; Haq et al., 2000; Pfister and Storey, 2002). The enzyme showed a high affinity for Kemptide

(**Figure 7**).The apparent Km value obtained with this peptide was 31 μM and Km value for Mg-ATP was 39 μM. These values are within the range reported for the enzyme from other sources (Denis et al., 1991; Mehrani and Storey, 1995) including the goldenrod gall insects (Pfister and Storey, 2002).

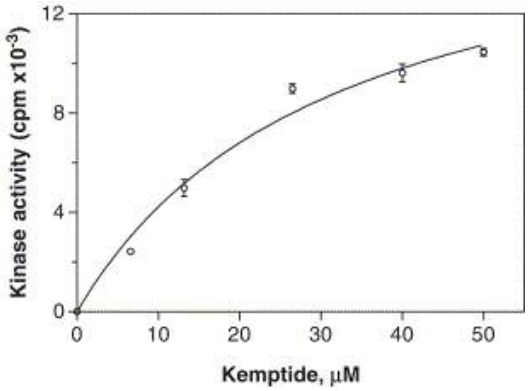


Figure 7. Concentration curve for Kemptide of Fat body PKA catalytic subunit activity. PKAc activity was measured in the presence of various concentrations of Kemptide, 0.2 mM ATP and 0.5mM magnesium acetate. Assays were performed at pH 7. Km value is 31 ± 7 μM.

Maximal activity was observed at pH 7 and 0.5mM Mg^{+2}. Higher concentrations of magnesium inhibited the purified enzyme ($IC_{50} \approx 5$ mM) (Table 2). A similar effect has been reported for kinases from different sources (Strålfors and Belfrage, 1982, Cao et al, 1995). It was of particular interest to examine whether Mn^{+2} could substitute Mg^{+2} as divalent cation, since drosophila catalytic subunit preferred manganese to magnesium for enzyme activity (Haracska and Udvardy, 1992). No kinase activity was found in the absence of Mg^{+2}. Mn^{+2} could not replace Mg^{+2} as divalent cation, but instead inhibited strongly the enzyme activity (**Table 2**). We also examined the effect of Ca^{+2} on the kinase activity since in adult *M. sexta* AKH increases intracellular calcium concentration and the processes mediated by Ca^{+2} are unknown (Arrese et al., 1999). The effect of

Ca^{+2} was tested on the activity of purified enzyme as well as in fat body homogenates. In both cases Ca^{+2} caused a strong inhibition of fat body kinase.

Table 2. Effect of divalent cations on the activity of purified PKA

	Relative activity		**Relative activity**
0.5 mM Mg^{+2}	*100*	0.5 mM Mn^{+2}	*0*
1 mM Mg^{+2}	*77.4±2.1*	0.5 mM Mn^{+2}+0.5 mM Mg^{+2}	*6.20±0.2*
5 mM Mg^{+2}	*39.1±0.2*	0.5 mM Ca^{+2}+0.5 mM Mg^{+2}	*2.50±0.1*

The PKA activity was assayed in the presence of 50 µM kemptide and 0.2 mM ATP. The values shown are the relative activities, compared with the value obtained for 0.50 mM Mg^{+2} (nominally 100%). Values represent the mean±SEM (*n*=3).

Histones serve as substrates for many serine/ threonine kinases and can be utilized as in vitro PKA substrate (Foster et al., 1984; Haq et al., 2000). We used histones as phosphate acceptor to monitor the purification. However, histones are a much poorer substrate for the insect kinase than Kemptide, and the Km value for histones was 0.73 ± 0.01 mM.

PKA inhibitors

The effect of different kinase inhibitors on the ability of the purified enzyme to phosphorylate Kemptide was investigated (**Figure 8**). The strongest inhibition was found with the synthetic peptide corresponding to residues 5-24 of mammalian inhibitor protein (PKI) that is a potent and specific inhibitor of PKA (Cheng et al., 1986). Approximately 90% of the enzyme activity was inhibited in the presence of 70 nM PKI 5-24 whereas complete inhibition was reached at 700

nM (**Figure 8**). The fact that a specific inhibitor of PKA produced complete inhibition of the kinase activity of purified PKA indicated that the presence of contamining kinases is highly unlikely. H-89 is also a very potent inhibitor of PKA (Ki 48nM) and we also found high inhibitory effect on the *M.sexta* enzyme being 70% of the activity inhibited by 700 nM. This effect is in the same range previously reported for other PKA kinases (Chijiwa et al., 1990).

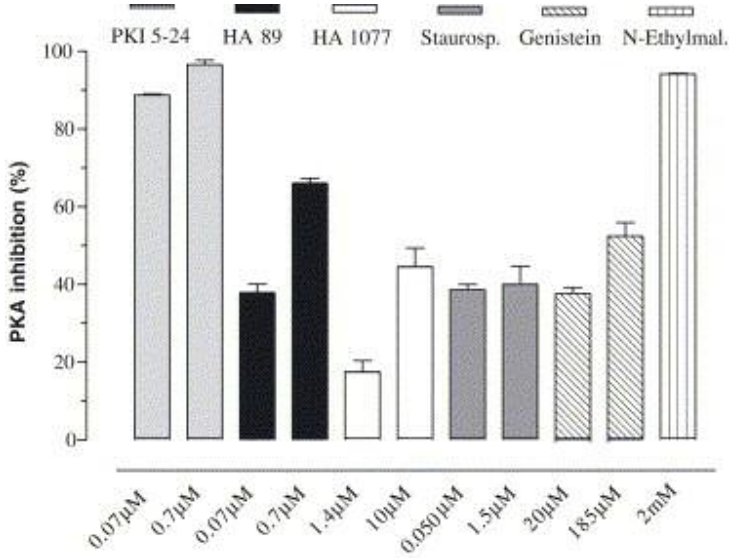


Figure 8. Effect of inhibitors on the activity of catalytic subunit of M.sexta fat body PKA. The percentage of inhibition was calculated using the activity in the absence of inhibitor. Results represent the mean ± SEM (n=3).

Staurosporine aglycon, which is a very potent PKC inhibitor (Fabre et al., 1993), partially inhibited PKA at 50 nM and no further inhibition was observed even at micromolar range (1.5μM). HA1077, a selective calcium/calmodulin protein kinase inhibitor (Takizawa et al., 1993) and genistein, a potent tyrosine kinase inhibitor (Akiyama et al., 1987) also showed a partial inhibitory effect only in micromolar range. Inhibitory effect of non-specific inhibitors at higher concentration is a common observation among kinases, as they are highly

conserved and recognize very similar substrate sequence (Taylor et al., 1990). We also examined the role of thiol groups in the enzyme activity by pre-incubating the enzyme with different concentrations of ethylmaleimide, a sulfhydryl group blocker. The activity decreased at increasing concentration of ethylmaleimide and complete inhibition was obtained at 2 mM. This result confirms that the catalytic activity of *M. sexta* PKA requires sulfhydryl groups as previously shown for PKA from other sources (Taylor et al., 1990; Haq et al., 2000).

Substrate specificity

In general kinases are classified into two groups, serine/threonine and tyrosine kinases. PKA catalyzes the transfer of the γ-phosphate from ATP to serine / threonine residues of substrate proteins (Taylor et al., 1990). We examined the amino acid specificity of the purified enzyme by comparing the ability of the enzyme phosphorylating different synthetic peptides containing serine, threonine or tyrosine as phosphate acceptor residue. These peptides have sequences that are specifically recognized by different types of kinases and also they are compatible with the simple assay in which binding to phosphocellulose is used to separate ^{32}P-labeled peptide from ^{32}P-ATP and ^{32}Pi. The threonine-containing peptide is a protein kinase C substrate derived from epidermal grow factor receptor (Heasly and Johnson, 1989) whereas tyrosine-containing peptide derived from pp60^{src} protein of Rous sarcoma virus (Casnelli et al., 1982) As shown in **Table 3**, *M. sexta* fat body PKA only recognized serine

residues as phosphate acceptor, neither threonine nor tyrosine containing peptides were phosphorylated. Like mammalian PKA, which recognizes a serine in the physiologically relevant protein substrates (Hjelmquist et al., 1974; Strålfors and Belfrage, 1983), the insect enzyme is also a serine kinase. We further examined substrate sequence specificity of the purified enzyme using synthetic peptides containing a serine residue flanked by different amino acids. The PKA recognition motif has been identified to be Arg-Arg-X-Ser- and this sequence is present in the Kemptide substrate. Two basic residues, usually arginine dyad, separated by a single residue from the phosphorylation site have been observed to be important for primary sequence recognition and substrate binding (Kemp et al., 1977; Feramisco et al., 1980; Denis et al., 1991). Replacement of either of these Arg, even by another basic residue is sufficient to increases Km. Replacement of Arg→Lys at –3 position increased Km by 90 fold, while Arg→Lys at –2 position increased Km by 16 fold of vertebrate kinase (Kemp et al., 1977). A basic residue at –6 position relative to phosphorylation site enhance mammalian enzyme activity, while it had negative effect on the yeast enzyme (Prorok and Lawrence, 1989; Denis et al., 1991). We tested two peptides containing single Ser. One of them has a Thr at -2 instead of Arg. This is Syntide 2 which is a specific substrate for calcium/calmodulin protein kinase (Hashimoto and Soderling, 1987) and proved to be a poorer substrate than Kemptide (**Table 3**). A similar observation was reported for the *Microsporum gypsum* PKA which phosphorylates Syntide 2 although at a lower rate than Kemptide (Haq et al, 2000).

Table 3. Substrate specificity of *M. sexta* fat body PKA

Substrate	Sequence	%
Kemptide	Leu-Arg-Arg-Ala-**Ser**-Leu-Gly	*100*
Thr-kinase	Lys-Arg-**Thr**-Leu-Arg-Arg	*0*
Tyr-kinase	Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-**Tyr**-Ala-Ala-Arg-Gly	*0*
Syntide 2	Pro-Leu-Ala-Arg-Thr-Leu-**Ser**-Val-Ala-Gly Leu-Pro-Gly Lys-Lys	*67.0±0.4*
PKC	Arg-Phe-Ala-Arg-Lys-Gly-**Ser**-Leu-Arg-Gln-Lys-Asn-Val	*159.5±6.9*

All peptides were assayed under the same conditions at a final concentration of 50 µM. Results are expressed as relative activity (%) to the activity obtained using Kemptide (0.39±0.05 µmol/ min-mg) and represent the mean±SEM (*n*=3). Underlined amino acids are phosphate acceptor.

The second peptide has a basic residue at -6 in addition to the basic residues at -3 and -2 positions. This peptide is a specific substrate for Protein kinase C (PKC) (House and Kemp, 1987). In this case a considerable higher kinase activity (159.5 ± 6.9 %) was obtained against this substrate compared to Kemptide. It seems that for the insect kinase the enhancement of activity provided by the basic residue at -6 position is more important than the replacement of Arg at -2. The activity of a vertebrate PKA – bovine PKA catalytic subunit from Sigma- was also compared to the insect PKA phosphorylating the PKC peptide. Interestingly, we also found that the activity of the mammalian kinase was higher against the PKC peptide (143 ± 8.3%) than Kemptide (100%). Altogether these results show that basic residues at -2 and -6 positions are important for the insect kinase activity. The fat body kinase recognizes substrate sequence similar to mammalian kinase compare to yeast.

The physiological substrates of *M. sexta* PKA are unknown. The components of the mechanism of TG mobilization in the insect fat body are potential targets of PKA action given the fact that AKH-induced lipid mobilization involves PKA activation (Arrese et al., 1999). The regulation of TG-lipase activity must be a critical point on the regulation of lipid mobilization. Previously we showed that TG- lipase is a phosphorylatable protein that can be phosphorylayed by the bovine catalytic subunit of PKA but the phosphorylation did not affect the lipase activity (Arrese and Wells, 1994). Because the possibility of having non-specific phosphorylations due to the use of bovine PKAc could not be ruled out in that experiment, a definitive conclusion was not possible. The catalytic subunit of PKA from the insect was required in order to verify that observation. For that purpose a preparation of fat body TG-lipase was incubated with purified kinase in the presence of [γ-^{32}P]ATP. The preparation was analyzed by SDS-PAGE followed by autoradiography. **Figure 9A** shows the SDS-PAGE in which the major band of 76kDa corresponds to the TG-lipase. An other minor component can also be seen, particularly a protein band of 50kDa. This contaminant was present in the TG-lipase preparation. We do not have information on the nature of this protein rather than it co-purified with the TG-lipase after several chromatographic steps. It is unknown whether this protein is in some way related to the role of the lipase.

The autoradiography (**Figure 9B**) shows that TG-lipase was phosphorylated *in vitro* by the purified catalytic subunit of PKA suggesting that the lipase could be a physiological substrate of this kinase

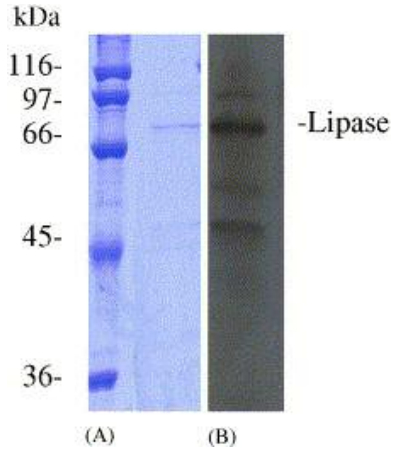


Figure 9. Phosphorylation of M.sexta fat body TG-lipase. Partially purified TG-lipase was incubated with [γ-^{32}P]ATP and purified catalytic subunit from M.sexta fat body. After incubation proteins were separated on 12% SDS-PAGE. Gel was stained with Coomassie and dried (A), and exposed for autoradiography (B) to visualize ^{32}P incorporation.

The enzymatic activity of the phosphorylated and unphosphorylated form of the enzyme was compared hydrolyzing [^{3}H]-triolein as micelles of Triton X-100. For this purpose two types of incubation were performed. On one hand TG-lipase was incubated with purified catalytic subunit of PKA in the presence (phosphorylated) and absence (control) of ATP as described in Material and Methods. Afterwards the lipase activity of both types of preparations was measured. We found that the phosphorylated and unphosphorylated (control) form of TG-lipase showed almost the same enzymatic activity hydrolyzing [^{3}H]-triolein as micelles of Triton X-100 (**Figure 10A**). The activity of phosphorylated lipase was slightly higher than the control (10 % increase) but this difference was not statistically significant. Panel 8-B shows the TG-lipase activity of fat body homogenates from control and AKH treated insects. As previously shown, AKH induced a significant increase -68%- in lipase activity of the homogenates.

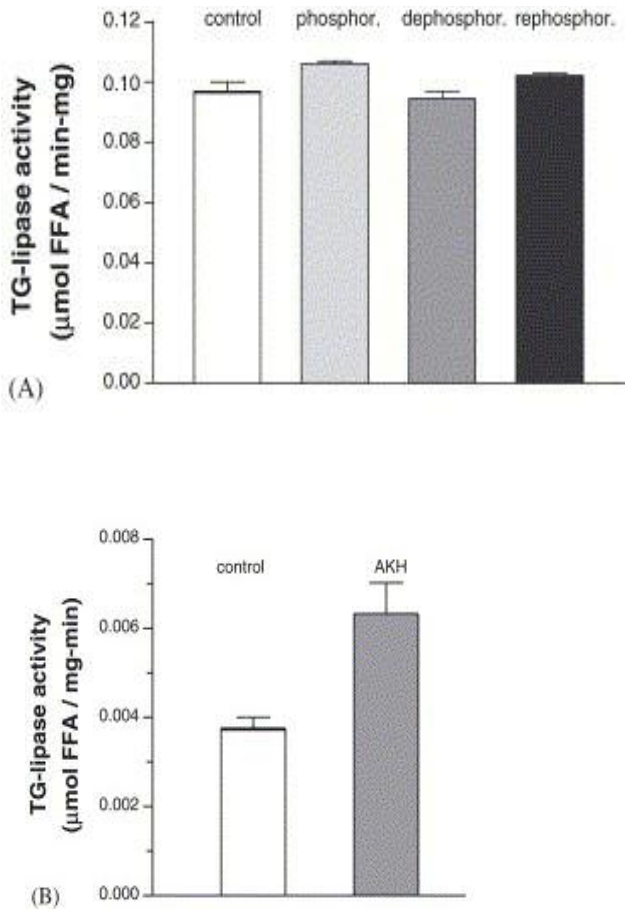


Figure 10. A) Effect of Phosphorylation on the Activity of TG Lipase. Partially purified lipase was phosphorylated using ATP in the presence of the catalytic subunit of *M. sexta* PKA. In "Control" samples, the lipase activity was determined using the enzyme as obtained after the purification. In "Phosphorylated" samples, the lipase was phosphorylated by PKAc prior to measure the lipase activity. In "Dephosphorylated" samples, the lipase was dephophorylated by alkaline phosphatase prior to measure the activity and for "Rephosphorylated" samples, the lipase was incubated with alkaline phosphatase followed by PKAc incubation before to measure the lipase activity. The activity hydrolyzing [^{3}H]-triolein was determined as described in Materials and Methods. Results represent the mean ± SEM (n=3). The difference of the means is not significant. B) Effect of AKH on the Activity of TG Lipase present in fat body homogenates. Results represent the mean ± SEM (n=3). The difference of the means is significant (P=0.026).

The fat body TG-lipase has several properties in common with the hormone-sensitive lipase - HSL - from the vertebrate adipose tissue (Arrese and Wells, 1994). Among those is that HSL is phosphorylated by the catalytic subunit of PKA from adipose tissue. However the *in vitro* phosphorylation of isolated rat

adipose tissue HSL with catalytic subunit of cAMP-dependent protein kinase results in an up to 3-fold increase in the lipase activity against triacylglycerol (Strålfors and Belfrage, 1983; Olsson *et al*., 1984). Several phosphorylation sites have been disclosed in HSL. Initially, phosphorylation of Ser 563 by PKA was identified as the regulatory site responsible for the phosphorylation-induced increase in hydrolytic activity. But PKA can also phosphorylate two other serines -659 and 660- and mutational analysis proved that phosphorylation of these two sites are responsible for *in vitro* activation of HSL. Also these sites are phosphorylated in adipocytes in response to stimulation of lipolysis. The role of phosphorylation of Ser 563 remains elusive. Apart from those three PKA phosphorylation sites, in quiescent cells HSL is phosphorylated *in vivo* at Ser 565 named the basal site. Other kinases phosphorylate HSL at the basal site and this impairs the phosphorylation of Ser 563 by PKA. However, the finding that Ser 563 is not essential for HSL activation raises some question about the antilipolytic role of Ser 565 phosphorylation (Holm *et al*., 2000). In any case the *in vitro* phosphorylation of HSL by PKA increases the hydrolytic activity of this enzyme hydrolyzing TG in an artificial substrate. Unlike the vertebrate lipase, PKA phosphorylation of the insect TG-lipase did not increase its hydrolytic activity. By analogy with HSL the insect lipase could have phosphorylated sites that impair the phosphorylation of regulatory sites. Even though the TG-lipase was purified from quiescent insects in which the enzyme is expected to be dephosphorylated the presence of previously phosphorylated sites on TG-lipase was examined. For this purpose, TG-lipase was incubated with alkaline

phosphatase to promote dephosphorylation, and subsequently it was phosphorylated by incubation with PKAc and ATP. Parallel incubations that were done in the presence of [γ-^{32}P] ATP and analyzed by SDS-PAGE followed by autoradiography showed the phosphorylation of the lipase (data not shown). The comparison of the activity of dephosphorylated and rephosphorylated lipase showed no significant differences (**Figure 10A**). Rephosphorylated lipase exhibited a slightly higher activity (8% increase) than the dephosphorylated lipase but this difference is not statistically significant. This result indicates that phosphorylation of TG-lipase does not constitute the main or, at least, the only step required for activation of lipolysis. Other proteins and/or mechanisms of activation must be also involved in the activation of lipolysis in the insect fat body.

CHAPTER-III

ACTIVATION OF THE LIPID DROPLET CONTROLS THE RATE OF LIPOLYSIS OF TRIGLYCERIDES IN THE INSECT FAT BODY

INTRODUCTION

Insects rely on lipid reserves to survive during physiological non-feeding periods, or to meet the energy requirements of developing eggs, flight, and starvation. The fat body is the principal site for storage of both glycogen and lipids. The fat body synthesizes most of the proteins found in the hemolymph, whereas also serving as the main storage site of triglycerides (TG), which constitute more than 90% of the fat body lipids. Therefore, functionally, the fat body accomplishes roles that in vertebrates are carried out by both liver and adipose tissue (Law and Wells, 1989).

The tobacco hornworm, *Manduca sexta*, feeds constantly and the content of fat body TG increases continuously until the end of the larval development. During the larval period, ~20 days, the content of TG in the fat body increases from a few µg on hatching to ~80 mg at the end of larval stage (Fernando-Warnakulasuriya et al., 1988). During subsequent development, the lipid reserves are used to sustain the life of the adult insect which feeds occasionally (Fernando-Warnakulasuriya et al., 1988; Ziegler, 1991; Arrese et al., 2001; Canavosa et al, 2001). Due to these metabolic features, *M. sexta* represents

an excellent model for studying the basic mechanisms involved in either the synthesis/deposition of TG in larvae or the mobilization of TG in adult insects (moth).

TG is stored in fat body adipocytes as cytosolic lipid droplets (Willott et al., 1988). TG hydrolysis (lipolysis) is mediated by a TG-lipase that has been purified from the cytosol (Arrese et. al., 1994). Like hormone sensitive lipase (HSL), which catalyzes the rate-limiting step in mobilization of adipose tissue fatty acids (Holm et. al., 2000; Kraemer et. al., 2002), the TG-lipase from *M. sexta* fat body is an enzyme that can be phosphorylated. The end-product of insect lipolysis is *sn*-1,2-diacylglycerol (DG) that is released into the hemolymph (Arrese et al., 1996; Arrese et al., 1997) and loaded into the hemolymph lipoprotein, lipophorin. This causes the transformation of high-density lipophorin (HDLp) into low-density lipophorin (LDLp), which transports DG to the sites of utilization e.g. the flight muscle, and ovaries, where it is hydrolyzed to fatty acids by a lipophorin-lipase (Soulages and Wells, 1994).

The lipolytic process is under hormonal regulation by the neuropeptide adipokinetic hormone, AKH (Gade and Auerswald, 2003). AKH action is comparable to that of glucagon in mammals. It contributes to hemolymph sugar homeostasis, and is also involved in the mobilization of sugar and lipids from the fat body during energy-requiring activities (Ziegler and Schulz, 1986a; Ziegler and Schulz, 1986b; Gade et al., 1997; Schoofs et al., 1997). AKH receptors from the fruit fly *Drosophila melanogaster* and the silkworm *Bombyx mori* have been recently identified (Staubli et al., 2002). These receptors are related to the

mammalian gonadotropin-releasing hormone receptor, which is a G protein-coupled receptor that activates both inositol phosphate and cAMP signaling responses. In *M. sexta*, AKH mobilizes glycogen during the larval stages and promotes a massive lipolytic response in the adult stage (Ziegler et al., 1990). AKH promotes a rapid activation of fat body cAMP-dependent protein kinase (PKA). Supporting the role of PKA in lipolysis, agents that raise intracellular cAMP concentration, such as 8Br-cAMp and forskolin, also stimulate lipolysis in the adult insect (Arrese et al., 1999). Besides the involvement of cAMP, the lipolytic response of AKH also induces a sustained increase in calcium influx (Wang et al., 1990; Lum and Chino, 1990; Arrese et al., 1999). Moreover, calcium mobilizing agents such as thapsigargin or ionomycin strongly stimulate lipolysis (Arrese et al., 1999). Therefore unlike the vertebrate system in which the lipolytic process is activated by cAMP (Honnor et al., 1985) and inhibited by intracellular calcium (Xue et al., 2001), in insects both messengers cAMP and calcium stimulate lipolysis. Protein phosphorylation mediated by PKA is expected to be part of the mechanism controlling the activation of lipolysis but the nature of the proteins targeted by PKA remains to be elucidated. It has been shown that TG-lipase can be phosphorylated *in vitro* by purified PKA from the insect fat body. However, this phosphorylation failed to increase the enzyme activity when assayed *in vitro* with an artificial substrate (Patel et al., 2004). In the present study the mechanism of activation of lipolysis was investigated using the native substrate of the TG-lipase, the lipid droplet, and a combination of *in vivo* and *in vitro* experiments. To study the role of PKA, and the impact of the

phosphorylation of the TG-lipase and the lipid droplet proteins, on the activation of lipolysis we used a novel approach in which the lipolytic process was reconstituted *in vitro* using *in vivo* TG-radiolabeled lipid droplets as the substrate of purified TG-lipase, and purified insect PKA. The studies presented here confirm the presumed role of PKA in activation of the lipolysis. More interestingly, the studies suggest that, contrary to previous assumptions, the main role of PKA is to activate the substrate of lipolysis, the lipid droplet, rather than the TG-lipase. The studies led to the identification of the "Lipid Storage Droplet Protein 1" as the main target of PKA activation as well as the main candidate to play a major role in the activation of lipolysis.

EXPERIMENTAL PROCEDURES

Materials: [^{32}P]Orthophosphate and [γ-^{32}P] ATP were purchased from MP Biochemicals (Irvine, CA). Labeled trioleoylglycerol ([tri-9,10-^{3}H(N)] oleoyl-glycerol) and [9,10 (n)^{3}H]-Palmitic acid were from PerkinElmer Life Sciences (Boston, MA). Protease and phosphatase inhibitors were purchased from Sigma-Aldrich (St. Louis, MO). DEAE Sepharose Fast Flow, Phenyl-Sepharose, and Q-Sepharose were from Amersham Pharmacia (Piscataway, NJ). Hydroxyapatite Bio-Gel HT Gel was purchased from BioRad (Hercules, CA) *M.sexta* adipokinetic hormone (AKH) was obtained from Peninsula Laboratories (Belmont, CA). Electrophoresis items were from Invitrogen (Carlsbad,CA). Silica gel G plates were purchased from J.T. Baker (Phillipsburg, NJ). Trypsin sequencing grade

was purchased from Promega (Madison, WI). All other chemicals were of analytical grade.

Experimental Insects: *M. sexta* eggs were purchased from Carolina Biological supplies (NC) and larvae were reared on artificial diet (Bell and Joachim, 1976). Adult insects were maintained at room temperature without food. All experiments were carried out using adult insects, 48-72h after emergence. To achieve a consistent basal level of lipolysis the insects were decapitated 24 h ahead of the experiment. Two hours before the experiments the insects were injected with 13 mg of trehalose dissolved in 20 μl of water (Arrese et al., 1996).

Purification of TG-lipase: TG-lipase was purified from the cytosolic fraction of *M. sexta* fat body homogenates using anion-exchange, hydroxyl-apatite, and hydrophobic interaction chromatography as reported previously (Arrese and Wells, 1994).

Purification of the Catalytic Subunit of PKA: The catalytic subunit of PKA was purified from the cytosolic fraction of *M. sexta* fat body homogenates using a combination of ion exchange chromatographies, as previously described (Patel et al., 2004).

Subcellular Fractionation: Fat body tissue from two insects was combined and homogenized with a Potter-Elvehjem glass homogenizer fitted with Teflon pestle,

using 6 ml of homogenization buffer (20 mM Tris, pH 7.4, 0.25 M sucrose, 1 mM EDTA, 0.1 mM benzamidine, 10 mg/l leupeptine, 1 mg/l aprotonin, 0.1% 2-mercaptoethanol, 2 mM imidazole, 2 mM sodium fluoride, 1.5 mM sodium molybdate, 1 mM sodium orthovanadate, and 4 mM sodium potassium tartrate). The homogenate was overlaid with 2 ml of buffer without sucrose, and centrifuged (100,000 x g for 1 hr). Three fractions were collected: fat cake, infranatant and pellet. To ensure that the lipid droplets were free of cytosolic components, the fat cake was resuspended in homogenization buffer and gently vortexed. The sucrose concentration was adjusted to 15% (w/v) and a layer of 2 ml buffer without sucrose was laid on top. Samples were centrifuged in SW40 rotor at 100,000g for 1 hr. Purified lipid droplets were collected from the top and resuspended in homogenization buffer without sucrose. Typically lipid droplets of two insect fat bodies were resuspended in 0.5 ml of buffer. The pellet was resuspended in buffer and re-centrifuged at 100,000 x g for 1 hr. The resulting pellet was dissolved in 1 ml of buffer and centrifuged at 500 x g for 15 min. The resulting supernatant was used as membranes fraction. The infranatant (cytosolic fraction) was passed through a small Q-Sepharose column equilibrated with buffer (10 mM Na_2HPO_4, pH-7.4, 1 mM EDTA, 0.1% 2-mercaptoethanol (v/v), 0.1 mM benzamidine 0.37 mM Triton-X-100, 10 mg/l leupeptine, 1 mg/l aprotonin, 2 mM imidazole, 2 mM sodium fluoride, 1.5 mM sodium molybdate, 1 mM sodium orthovanadate and 4 mM sodium potassium tartrate). The column was extensively washed with equilibration buffer and the proteins eluted with a NaCl gradient (20 mM-150 mM) in the same buffer. TG-lipase was eluted with

180 mM NaCl in the same buffer and all the fractions containing TG-lipase activity were pooled, dialyzed and concentrated to a final volume of 4 ml.

In vivo Protein Phosphorylation Studies: Experimental insects were injected with 250 μCi of [^{32}P] orthophosphate (carrier free), and 90 min later with 100 pmol of AKH. Fat body tissue was dissected at various times after the hormonal injection. For each time, tissue from two insects was pooled and homogenized. The lipid droplets were isolated as indicated above. The lipid droplet associated proteins were separated by SDS-PAGE on 10% gels according to Laemmli, but in sample buffer containing 6% (w/v) SDS. Proteins were stained with Coomassie Blue. The gel was dried and phosphorylation was visualized by autoradiography. Gels and autoradiograms were scanned on an imaging densitometer (BIO-RAD model GS-700). The intensity of protein bands and phosphorylation were quantified from the gel and autoradiogram scans, respectively, using the Multi Analyst Macintosh software.

Preparation of Endogenously [^{3}H]-TG-Labeled Lipid Droplets: Fat body lipids were radiolabeled following a long-term procedure previously reported (Arrese and Wells, 1997; Arrese et al., 2001). Briefly, during the fifth larval instar, insects were fed 200 μCi of [9,10(n) ^{3}H]-Palmitic acid and, after completion of development (32 days), adult insects were decapitated and injected with trehalose as indicated above. Lipolysis was stimulated by injection of 100 pmol of AKH whereas injection of buffer provided the level of basal lipolysis. Fat bodies

were dissected 20 minutes after injection and the lipid droplets isolated as described above. Lipid analysis of TG showed that the vast majority of the radiolabel (99.8%) was localized in the fatty acyl residues. The remaining 0.2% was found in the glycerol backbone. sn-1,3 positions of TG contained 88.4±2% of the label. Analysis of the distribution of radioactivity among different neutral lipid classes of the lipid droplets showed that TG contained 97.4±0.5% of the radiaoctivity.

TG-lipase Activity Against [^{3}H]-TG-lipid droplets: An aliquot of the lipid droplet preparation containing 100 nmoles of TG was transferred to a glass tube containing lipase reaction buffer. The reaction was initiated by adding purified TG-lipase (7 µg) in a final volume of 150 µl. Final reaction conditions were 50 mM Tris, pH 7.9, 500 mM NaCl, 0.02 % (w/v) BSA, 2 mM dithiothretiol, 0.67 mM TG, and 0.37 mM Triton X-100. The mixture was gently vortexed for 20 sec and incubated at 37°C with constant shaking. After 30 min the reaction was terminated by the addition of 750 µl of chloroform: methanol (2:1) and 5 µl of 6 N HCl. The mixture was vortexed for 1 minute, and centrifuged at 2000 g for 2 minutes. The organic phase was collected, and cpm counted in an aliquot. The remaining organic phase was dried under a stream of nitrogen and the lipids separated by thin-layer chromatography on silica gel G plates using hexane: ethyl ether: formic acid (70:30:3) as the developing solvent (Arrese et al., 2001). The MG, DG, FFA and TG fractions were visualized by I_2 vapors and scraped from the plates. After complete removal of the I_2 the radioactivity associated with

each fraction was determined by liquid scintillation counting. Blank reactions, in which the TG-lipase was omitted, were used to obtain the basal level of distribution of radioactivity among the lipid classes and calculate the percentage of hydrolysis. The lipase activity was expressed as nmol of TG hydrolyzed/min-mg protein. All determinations were carried out in duplicate. Aliquots of lipid droplets resuspended in TG-lipase reaction buffer were freshly stained with Oil Red O and were observed under the microscope. We found numerous red-colored spheres (0.6–3.5 μm) confirming the existence of the lipid droplets in the reaction mixture. The size of lipid droplets in the final preparation was slightly smaller than the size of native lipid droplets (1.2 - 4.1 μm).

In Vitro Phosphorylation Reactions of TG-Lipase and Lipid Droplets: The lipid droplets were phosphorylated in a reaction mixture (90 μl) containing kinase reaction buffer (50 mM MOPS, 1 mM magnesium acetate, 0.5 mM EDTA and 2 mM dithiothreitol), purifed PKA (0.25 units), 7 μl lipid droplets (0.1 μmol TG), and 0.2 mM [γ-^{32}P]ATP (5 x 10^6 cpm / nmol). After 20 min of incubation at room temperature, the reaction was terminated by addition of electrophoresis sample buffer and analyzed by SDS-PAGE on 4-20% acrylamide gels followed by autoradiography. [^{32}P]-labeled protein bands profile was performed by densitometric analysis.

Purified TG-lipase was phosphorylated by insect PKA in the presence of [γ-^{32}P]ATP as described above. Phosphorylation of the lipase was confirmed by SDS-PAGE and autoradiography.

To study the effect of phosphorylation on the TG-lipase activity, [^{3}H]-TG-lipid droplets and/or TG-lipase were phosphorylated as described above, but using unlabeled ATP. The reactions were terminated by addition of 5 mM EDTA.

Measurement of LDL / HDL ratio of hemolymph: The content of DG in the hemolymph was assessed by measuring the relative mass of low-density lipophorin (LDLp) and high-density lipophorin (HDLp) after separation of the lipoproteins by ultracentrifugation in a KBr density gradient (Arrese et al., 1996). The LDLp / HDLp ratio was used as an index of lipolysis.

Western blotting: Polyclonal antibodies against purified TG-lipase were raised in chicken at Cocalico Biologicals (Reamstown, PA). For Western Blotting, proteins were separated by SDS-PAGE (10%) and transferred to nitrocellulose membranes. Immunodetection was performed using anti-TG-lipase antibodies (1:200). After incubation of membranes with horseradish peroxidase-conjugated rabbit anti-chicken secondary antibody (1:50,000), peroxidase activity was detected using ECL chemiluminescence reagents (Amershan Pharmacia, NJ). X-ray films were scanned and the intensity of the positive signal was quantified by densitometry.

Protein and TG Content of Lipid Droplets: The lipid droplet-associated proteins were precipitated with acetone (85% v/v) at -20ºC for at least 2 h. Afterwards samples were centrifuged at 10,000 x g for 5 minutes and the

resultant pellet was dissolved in final 10% SDS (w/v). An aliquot was used to measure total protein using the BCA method (Pierce, Rockford, IL).

TG was determined using the Infinity Triglyceride reagent kit as described by the manufacturer (ThermoTrace Ltd, Melbourne, Australia). Triolein was used as standard for the calibration curve.

Statistics: Statistical comparisons were made by the Student's t-test; $P<0.05$ was considered to be significant.

RESULTS

Study of a potential translocation of the lipase from the cytosol to the lipid droplet

Studies carried out in rat and 3T3 L1 adipocytes have shown that activation of lipolysis triggers the translocation of HSL from the cytosol to the substrate contained in the lipid droplets (Egan et al., 1992; Brasaemle et al., 2000). The potential role of this mechanism of lipolysis activation in *M. sexta* fat body was investigated by determining the relative distribution of TG-lipase between the cytosolic and lipid droplet fractions of the adipocytes. Immunoblotting was used to estimate the abundance of TG-lipase in the fractions. A comparison between the fractions obtained from control insects, and insects treated with AKH for 20 min indicated that activation of lipolysis does not change the levels of cytosolic TG lipase (**Figure 11**). Moreover, we were not able to detect the presence of TG-lipase in the lipid droplet fractions. Regardless of the lipolytic condition, the fat

body TG-lipase was exclusively found in the cytosol, and its abundance was unaffected by the level of lipolysis (**Figure 11**).

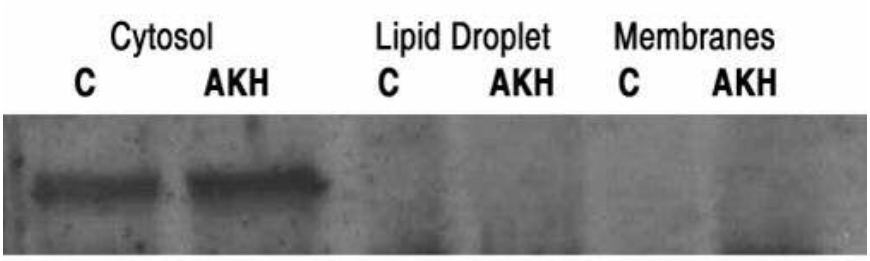


Figure 11. Subcellular distribution of TG-lipase among cytosol, lipid droplet, and membranes fractions under basal and stimulated lipolysis. Cytosol, membranes and lipid droplets from the fat body of control insects (C) and AKH-stimulated insects (AKH) were separated on 8 % SDS-PAGE and analyzed by Western blotting using anti-serum against TG-lipase. Normalization among control and stimulated samples for each subcellular fraction was accomplished loading equal percentages of each fraction onto each lane (0.5% of total for cytosol, 2% of total for lipid droplets, and 0.4% for membranes).

Effect of TG-lipase phosphorylation on the enzyme activity against TG contained in the native substrate, the lipid droplet

Previous studies from our laboratory have shown that PKA phosphorylates the TG-lipase (Arrese and Wells, 1994), but does not enhance the lipase activity (Patel et al., 2004). Because these studies were carried out using an artificial emulsion of TG that certainly does not have the structural and chemical properties of the lipid droplets, we investigated the role of the phosphorylation using the native lipase substrate, the lipid droplets. To obtain lipid droplets highly radiolabeled in the TG moiety *M. sexta* larvae were fed with [^{3}H]-palmitic acid. Because the moths emerge one month after the larvae were fed there is a nearly identical distribution of radioactivity and mass among the lipid species (Arrese et al., 2001). The activity of purified lipase, and *in vitro* PKA-phosphorylated lipase

were determined using lipid droplets isolated from the adipocytes of control insects (basal lipolysis) and from AKH treated insects (20 min after injection).

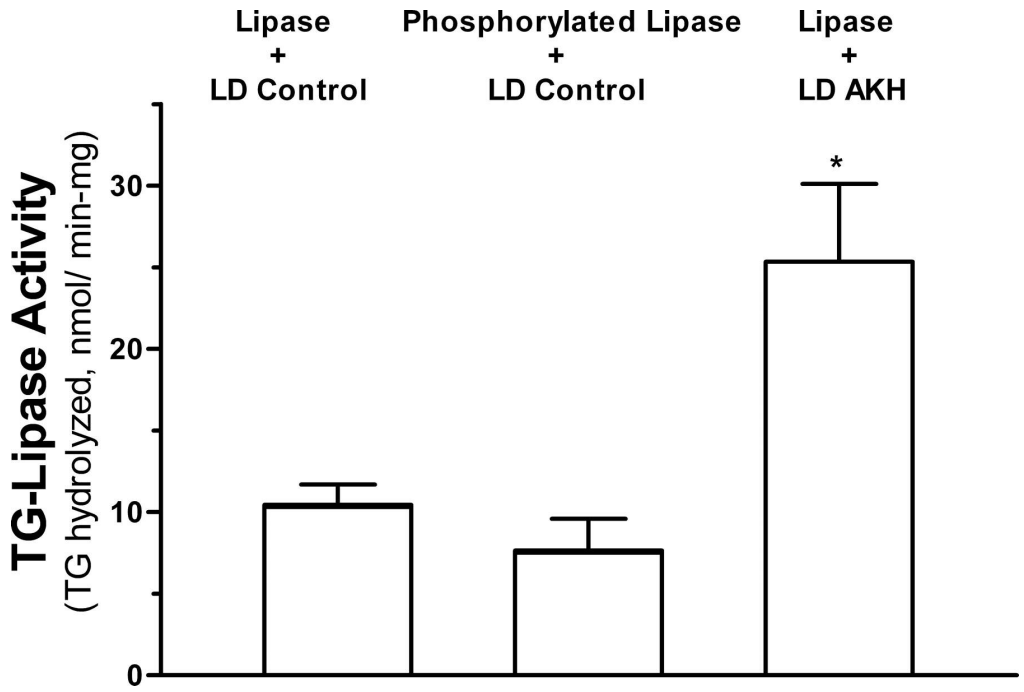


Figure 12. Effect of TG-lipase phosphorylation and AKH induced changes in the substrate on the lipase activity hydrolyzing TG present in the lipid droplets (LD). Purified TG-lipase from fat body tissue was *in vitro* phosphorylated by incubation with fat body cAMP-dependent protein kinase (PKA). The activity of purified TG-lipase was measured against i*n vivo* radiolabeled ([^{3}H]-TG)-lipid droplets isolated from insects with basal lipolysis (LD control). The effect of AKH on the substrate properties was studied using lipid droplets isolated from insects with stimulated lipolysis (20 min after injection of AKH) (LD AKH). Enzyme activities are expressed in nmol TG hydrolyzed/ min mg protein. Data represent the mean ± SEM (n=4). *P<0.05 vs control lipid droplets.LD: Lipid Droplet.

Phosphorylation of the lipase did not modify the enzyme activity hydrolyzing TG contained in the lipid droplets from control insects (**Figure 12**). However, the activity of the lipase against lipid droplets isolated from AKH treated insects was significantly greater (2.4 fold-increase) than that measured against lipid droplets obtained from control insects (**Figure 12**).

It must be noted that no significant endogenous lipase activity was detected in lipid droplets from control or AKH treated insects. This was determined by incubating the lipid droplets in the reaction buffer but in the absence of TG-lipase for different time periods (0 to 60 min). This result is consistent with the absence of lipase associated to the lipid droplets inferred from the Western Blotting analysis (**Figure 11**).

Time course of AKH-induced activation and phosphorylation of the lipid droplets

AKH-induced activation of lipolysis involves a rapid 4-fold increase of fat body PKA activity that is reached 2 to 5 min after the injection of the hormone into the hemolymph (Arrese et al., 1999). Potential changes in the phosphorylation state of the proteins of the lipid droplets and its possible correlation with the "activity" of the lipid droplets against purified TG-lipase were investigated. To study the time course of changes in protein phosphorylation together with the lipase activity measured against TG contained in the lipid droplets, we used lipid droplets containing both radiolabeled TG and ^{32}P-radiolabeled proteins isolated from insects fed with [^{3}H]-palmitic acid and injected with ^{32}P-orthophosphate 90 min prior to the experiments.

As shown in the **Figure 13**, the activity of purified TG-lipase was dependent on the time of isolation of the lipid droplets. Rapid and significant changes in the sensitivity of the lipid droplets towards the lipase were observed as early as 5 min after the hormonal stimulation. The highest lipase activity (2.6 fold-increase

over basal) was observed against the lipid droplets that were isolated 10 min after the stimulation of lipolysis.

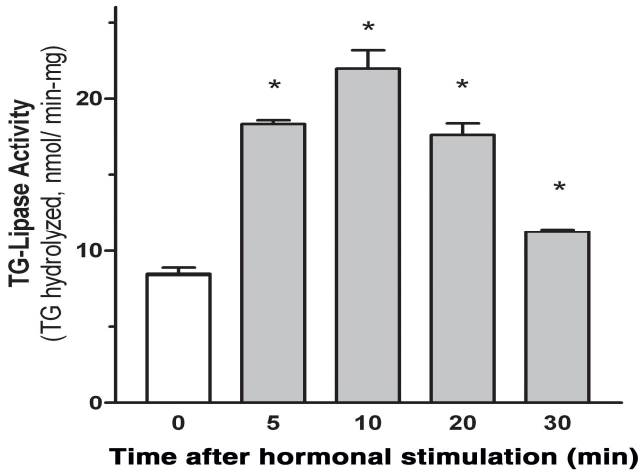


Figure 13. Time course of the activation of the substrate induced by AKH on the activity of TG-lipase. [^{3} H]-TG lipid droplets were isolated from insects with basal lipolytic activity (0) and AKH-stimulated insects (5, 10, 20, and 30 min after treatment). Aliquots containing 100 nmol of TG were used to measure the TG-lipase activity. Incubations were done for 30 min at 37 ºC as indicated in Experimental Procedures. Data are expressed in nmol TG hydrolyzed/ min mg protein and represent the mean ± SEM (n=4). *P < 0.05 vs. control.

^{3}H/^{32}P-radiolabeled lipid droplets were also used to examine the time dependent changes in the pattern of [^{32}P]-protein phosphorylation. **Figure 14A** depicts the protein profiles of lipid droplets separated in 10% SDS-PAGE. The autoradiogram of the same SDS-PAGE gel is shown in the panel B. Five minutes after the stimulation of lipolysis the only significant change in phosphorylation were observed in a band of ~43 kDa (2.7-fold increase). The change in phosphorylation of the 43 kDa band is very rapid reaching a maximum 10 min after the injection of the hormone (**Figure 15A, B and C**). Densitometric analysis of the gel indicated that the 43kDa band of the autoradiogram corresponded to

two close protein bands migrating with apparent masses of 42.8 and 44.2kDa (**Figure 14B**).

Figure 15D shows that the changes in phosphorylation 42/44 kDa band are paralleled by the changes in the sensitivity of the lipid droplets to TG-lipase. The increase in the level of phosphorylation of this band correlated (r^2 = 0.75) with the increase in TG-lipase activity.

Changes in the phosphorylation of other protein bands with apparent masses of 52, 66, 95, and 110 kDa were also observed. However, no correlation was found with the phosphorylation of these proteins and the lipase activity. For instance, phosphorylation of the 52, 66 and 110 kDa bands became more relevant at 20 and 30 min.

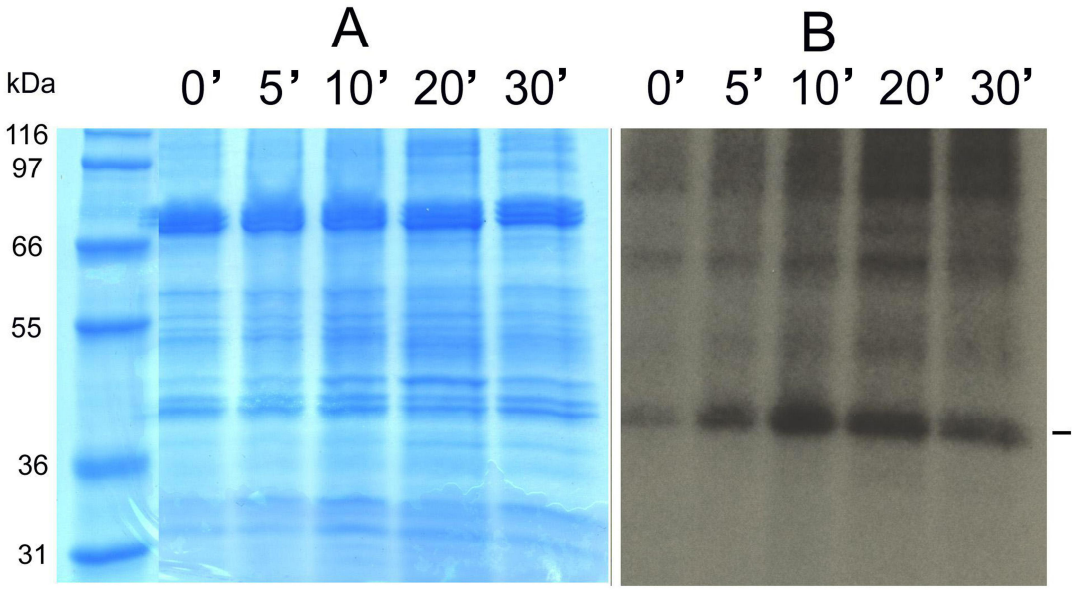


Figure 14. Time dependent changes in phosphoproteins of the lipid droplets. [^{32}P]-Lipid droplets were isolated at different times after hormonal treatment and subjected to SDS-PAGE in 10% acrylamide gels. A) Coomassie blue stained gel; B) Autoradiogram of the gel shown in A. Control lipid droplets were loaded in lanes labeled 0. The remaining lanes were loaded with lipid droplets isolated 5, 10, 20 and 30 min after the injection of AKH, as indicated in the figure. Approximately 15µg of protein was loaded into each lane. The intensity of protein bands and phosphorylation were quantified from the gel and autoradiogram scans, respectively, using an imaging densitometer as explained in Experimental Procedures. The phosphorylation state was calculated as the ratio between the intensity of phosphorylation and the intensity of the protein band.

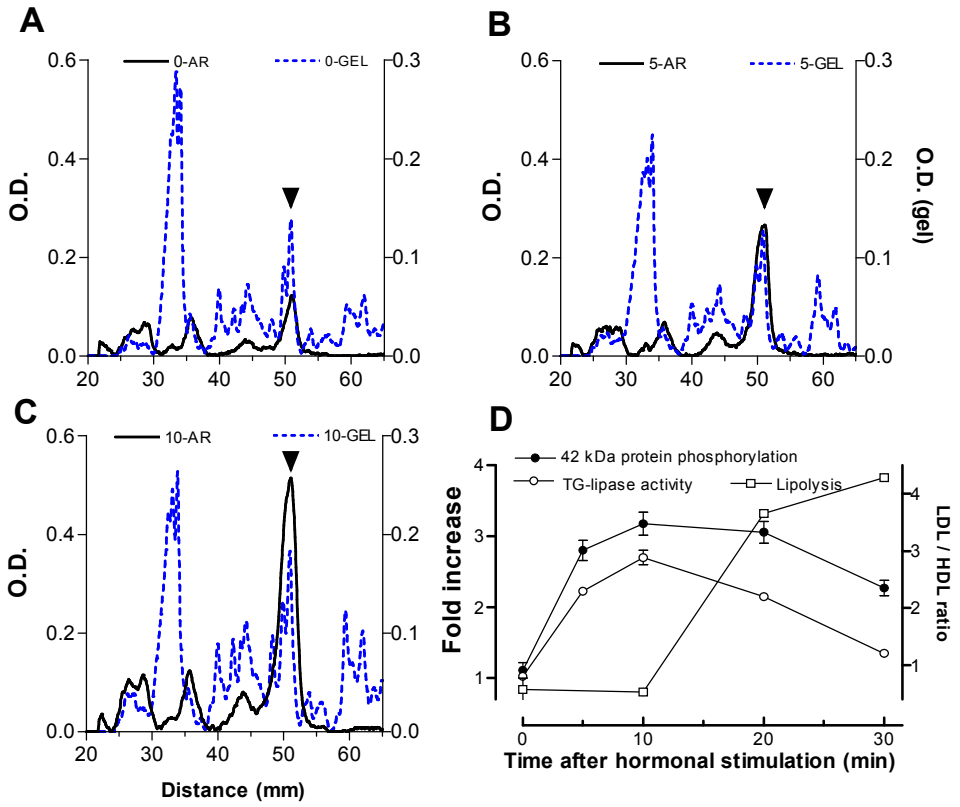


Figure 15. Correlation between the phosphorylation of the lipid droplets and their activity as TG-lipase substrates. A-C) show the gel and autoradiogram scans corresponding to control (0), 5 and 10 min lipid droplets shown in **Figure 14**. The major phosphoprotein is 42 kDa (52 mm). Other phosphoproteins are : 52 kDa (43 mm), 66 kDa (36 mm), 95 kDa (28 mm) and 110 kDa (26 mm); D) The time course of the AKH-induced changes in substrate activity showed in **Figure 14** are correlated with the time dependent changes in the phosphorylation state of the 42 kDa lipid droplet-associated protein. The phosphorylation state and the lipase activity were expressed as fold increase over the basal condition. The ratio of low-density lipophorin (LDLp) to high-density lipophorin (HDLp) is proportional to the level of circulating diacylglycerol (DG). The increase in the LDLp/HDLp ratio is the consequence of AKH induced lipolysis. Data are the mean ± SEM; n=4 for lipase activity, n=3 for phosphorylation of 42 kDa protein and LDLp/HDLp ratio.

As expected, the changes in lipase sensitivity of the lipid droplets preceded the lipolytic response measured by the increase in the content hemolymph DG that was estimated as the ratio of hemolymph low- and high-density lipophorin (LDLp and HDLp, respectively) (**Figure 15D**). In these insects the onset of lipid mobilization from the fat body into the hemolymph begins 10 min after the injection of 100 pmol of AKH (Arrese et al., 1996).

The highly phosphorylated 42/44 kDa band was identified as “Lipid Storage Droplet Protein 1” (Lsdp1) in the laboratory. In brief, the ^{32}P-radiolabeled proteins were separated by two-dimentional electrophorosis. The highly phosphorylated 42/44 kDa protein band was excised and subjected to identification by liquid chromatography mass spectometry/mass chromatography at Harvard Microchemistry Facility. For more details refer Patel et al. 2005.

In Vitro activation of lipolysis from purified TG-Lipase, PKA, and lipid droplets

The phosphorylation profiles of the lipid droplet-associated proteins obtained upon incubation of lipid droplets isolated from control insects (basal lipolysis) with purified fat body PKA in the presence of [γ-^{32}P]-ATP were compared to the phosphorylation profiles observed *in vivo* (**Figure 16**). This comparison showed similarities between the pattern of proteins phosphorylated *in vitro* by PKA and the pattern of phosphorylation observed *in vivo* when the lipolysis is stimulated by AKH. As observed in *in vivo* experiments, Lsdp1 was also the major phosphoprotein of the lipid droplet after *in vitro* phosphorylation indicating that Lsdp1 is the main substrate of PKA in the lipid droplets.

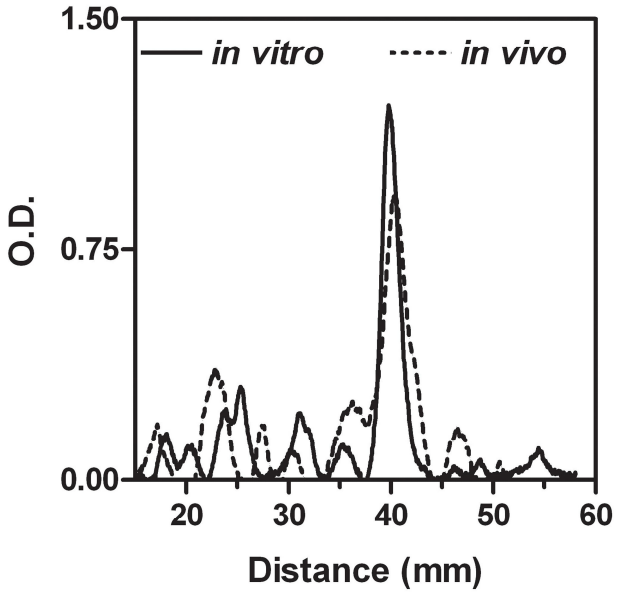


Figure 16. Comparison between the patterns of protein phosphorylation of the lipid droplets observed *in vivo* (AKH induced) and *in vitro* (PKA-catalyzed phosphorylation). Densitometric profiles of the autoradiograms of ^{32}P-phosphoproteins of the lipid droplets. *In vivo* phosphorylation was induced by AKH injection into insects previously injected with 250 μCi of [^{32}P] orthophosphate. Lipid droplets were isolated after 20 min of AKH treatment. For *in vitro* phosphorylation, lipid droplets were isolated from control insects (basal lipolysis) and incubated with purified cAMP-dependent protein kinase (PKA) and 0.2 mM [γ-^{32}P]-ATP (5 x 10^6 cpm / nmol) for 20 min. Both samples were subjected to SDS-PAGE in 4-20% acrylamide, which provides a good resolution in a broad range of size (6 to 200 kDa). Autoradiograms were scanned on an imaging densitometer as explained in Experimental Procedures. The major peak corresponds to the 42 kDa band.

In order to study the role of PKA on the activation of lipolysis, [^{3}H-TG]-lipid droplets (from insects with basal lipolysis) were incubated with or without purified PKA and ATP. Following phosphorylation the lipid droplets were incubated with purified lipase and the enzyme activity determined. The results showed that *in vitro* phosphorylation of the lipid droplets with PKA promotes a significant enhancement, 2-fold, of the lipase activity (**Figure 17**).

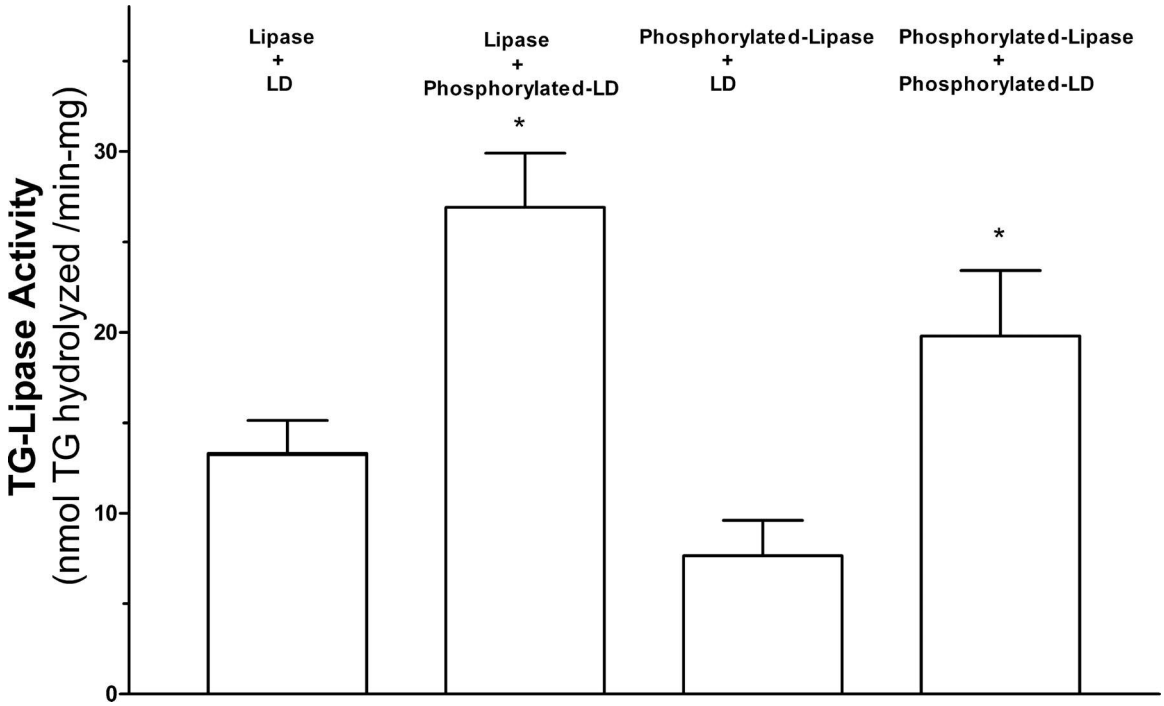


Figure 17. Effect of cAMP-dependent protein kinase (PKA) mediated phosphorylation of lipid droplet (LD) proteins on the activity of TG-lipase and phosphorylated TG-lipase. *In vivo* radiolabeled ([^{3} H]-TG) lipid droplets (LD) were isolated from insects with basal lipolytic activity and incubated 20 min with 0.2 mM ATP in the presence of purified PKA (Phosphorylated-LD) or in the absence of PKA (Control LD). The reaction was terminated by the addition of 5 mM EDTA (final concentration). Subsequently the samples were used as the substrate for both TG-lipase and phosphorylated TG-lipase. Purified TG-lipase was phosphorylated *in vitro* by incubation with purified PKA and ATP. Data that are expressed in nmol TG hydrolyzed/ min mg protein represent the mean ± SEM (n=4). * $P < 0.05$ vs LD control.

The activity of PKA phosphorylated TG-lipase was also assessed against basal and phosphorylated lipid droplets. As previously shown with *in vivo* activated lipid droplets (**Figure 12**), the state of phosphorylation of the lipase did not increase the enzyme activity, even in the presence of phosphorylated lipid droplets (**Figure 17**). It is concluded that under these conditions the difference in the hydrolytic capacity of the enzyme is due to the phosphorylation state of the TG substrate and is unaffected by the phosphorylation state of the lipase.

DISCUSSION

Understanding the regulation of the lipolytic process is essential to the full understanding of the metabolism of triglycerides. The mechanism of basal lipolysis and the mechanism of activation of lipolysis are complex processes whose details are far from being fully understood in any system (Londos et al., 1999; Holm, 2003). The complexity of the lipolytic activation is given by the fact that the lipolytic process involves the interaction of an enzyme, whose properties are not well established, with a lipid surface whose structure is not well understood, either. The lipid droplet is recognized as an important organelle which accomplishes essential functions in adipocytes and therefore in the homeostasis of lipid metabolism of the organism. The lipid droplets contain a large number of proteins embedded in the lipid surface (Brasaemle et al., 2004; Liu et al., 2004). Many of these proteins could play an active role in the lipolytic response and other metabolic reactions. The importance of studying these proteins is currently recognized. However, none of the potentially important proteins have been fully characterized from a functional or structural point of view, yet. Most of the current understanding of the lipolytic process has been achieved from studies carried out in 3T3 adipocytes (Londos et al., 1999; Holm, 2003). The current knowledge of lipolysis in insects is far more modest than that achieved in mammalian cells. The current study provided several advances on the mechanism of lipolysis in insects. Moreover, the experimental conditions for an *in vitro* system that allows the reconstitution of the lipolytic activation were established in this study. This *in vitro* system combined with the simplicity of

carrying out *in vivo* studies represents an important advance that will facilitate future studies on the molecular mechanism underlying the mobilization of TG stores in insects.

Substrate activation plays a major role in the activation of lipolysis

Our puzzling inability to observe phosphorylation-dependent activation of the lipase using an artificial substrate (Patel et al., 2004), led us to study the role of phosphorylation of the lipase using the native substrate, the lipid droplets. We thought that the artificial substrate, an emulsion of TG and TritonX-100 (1:5, mol/mol), prevented the observation of the activation of the lipase upon its phosphorylation. The ability to obtain homogenously TG-radiolabeled lipid droplets allowed the study of the role of lipase phosphorylation under experimental conditions that resembled the physiological conditions. Even in this condition phosphorylation of the lipase did not promote an increase in the lipolytic rate. However, the use of the native substrate led us to the main finding of this work which is that hormonal stimulation of TG lipolysis in insects involves the activation of the TG substrate, the lipid droplets. The fact that the activity of purified lipase was greater against lipid droplets isolated from insects with high levels of lipolytic activity than against lipid droplets isolated from insects in a basal lipolytic state (**Figure 12** and **13**), provided the first evidence indicating that the activation of the substrate was an important factor in the rate of lipolysis. The activation of the substrate could represent the major point of control of the

lipolytic rate. This information provides new evidence to the increasing notion that the lipid droplets, as organelles, are active participant in the process of lipolysis.

Phosphorylation of the substrate activates the lipolysis

As shown in **Figure 14**, hormonal activation of lipolysis alters the pattern of phosphorylation of the lipid droplets. The changes in phosphorylation are very fast and have a time course that correlates to a good extent with the activation state of the lipid droplets (**Figure 15**). These observations suggested a relevant role of substrate phosphorylation in the activation of lipolysis. The importance of the phosphorylation of the lipid droplets in the activity of the lipase was clearly demonstrated when the activation of the lipid droplets was achieved in the reconstituted system containing the purified enzymes, PKA and lipase, and control lipid droplets (Figure 17). This *in vitro* assay allowed discarding the potential influence on the rate of lipolysis of other changes in structure and composition of the lipid droplets that could take place *in vivo*. Moreover, it proved that the combination of TG-lipase, PKA, and lipid droplets, without additional cellular machinery, reproduce to a great extent the activation observed with lipid droplets obtained from insects treated with AKH.

Role of Lsdp-1 phosphorylation in the activation of lipolysis

Inspection of the pattern of phosphorylation of the proteins of the lipid droplets showed that a 42-44 kDa protein was the main target of the phosphorylation cascade triggered by AKH. On the basis of its sequence identity with a full-length

sequence from *Drosophila melanogaster*, this protein was identified as Lipid storage droplet protein 1 (Lsdp1). Lsdp1 is not highly abundant in the lipid droplets of adult insects; however it is the main phosphoprotein. Lsdp1 was the prevalent target of *in vivo* phosphorylation, induced by AKH, as well as the main target of PKA when the lipid droplets are phosphorylated *in vitro.* Moreover, since the changes in phosphorylation of Lsdp1 correlated with the "activity" of the lipid droplets measured with purified lipase, the present studies suggest that Lsdp1 could play a major role in the activation of TG lipolysis in the insect fat body. This study did not provide information on the role of the phosphorylation of the other phosphoproteins observed in the lipid droplets.

Role of PKA in the activation of lipolysis

Previous studies have shown that AKH promotes an increase in PKA activity, as determined by activation of glycogen phosphorylase (Gade and Auerswald, 2003). On the other hand, supporting a role for PKA in the activation of lipolysis, other studies have shown that cAMP analogues and adenylate cyclase activators activate lipolysis (Arrese et al., 1999). Although these previous studies suggested a role of PKA in the activation of lipolysis, they did not provide direct experimental evidence linking PKA and lipolysis. The present study provided direct evidence supporting the role of PKA in activation of lipolysis. It showed that the pattern of *in vitro* phosphorylation of the lipid droplets with purified PKA is very similar to that observed upon *in vivo* stimulation of the insects with AKH

(**Figure 16**) and that the lipolytic activation can be reconstituted *in vitro* from isolated lipid droplets and purified PKA and lipase (**Figure 17**).

In vitro phosphorylation of TG-lipase catalyzed by PKA did not enhance its activity against the native (**Figure 12**) or the artificial substrate (Patel et al., 2004).This is a clear difference with the mammalian HSL that PKA *in vitro* phosphorylation induced a 3-fold increase of its activity (Stralfors and Belfrage, 1983; Olsson et al., 1984).

Role of lipase binding to the lipid droplet and kinetics of TG hydrolysis on the activation of lipolysis

Hormonal stimulation of the lipolytic response in mammalian adipocytes induces the phosphorylation and translocation of HSL to the surface of the lipid droplet. This mechanism is the first critical step of lipolysis and explains at least partially the activation of lipolysis in mammalian adipocytes (Egan et al., 1992; Londos et al., 1999; Brasemle et al., 2000; Holm, 2003). We investigated a potential role of the translocation of the fat body lipase to the lipid droplet in the activation of lipolysis. We did not find evidence indicating the presence of the lipase in the lipid droplet of adipocytes obtained from insects with either basal or high lipolytic rates. This result indicates that activation of lipolysis in insects does not involve a tight association of the TG-lipase with its substrate, the lipid droplets. In other words, translocation of the enzyme to the lipid droplet does not seem to take place or, if it does, it involves a low binding affinity association, such that the enzyme would dissociate from the lipid droplet during the preparation of the

homogenate and centrifugation. However, our studies demonstrated that activation of lipolysis does not involve a high affinity binding of the cytosolic lipase to the lipid droplet. Therefore, a change in the kinetic properties of the lipase could be responsible for the enhancement in the lipase activity that is observed when the lipid droplets are phosphorylated with PKA *in vitro* or, when the lipid droplets are isolated from AKH stimulated insects. This increase in the catalytic activity could be due to an increase in the accessibility of the lipase to the TG molecules. This study suggests that the investigation of the role of the structure and phosphorylation of Lsdp-1 in the accessibility of TG to the lipase could provide relevant information about the mechanism of activation.

Concluding remarks

This study provided direct evidence on the role of the lipid droplets in the activation of lipolysis in fat body adipocytes. The reconstitution of the lipolytic activation *in vitro* demonstrated a prominent role of PKA mediated phosphorylation of the lipid droplets in the lipolysis. Phosphorylation of Lsdp1 emerges as a likely regulator of the lipolytic activation

CHAPTER-IV

ADIPOKINETIC HORMONE-INDUCED MOBILIZATION OF FAT BODY TRIGLYCERIDE STORES IN *MANDUCA SEXTA*: ROLE OF TG-LIPASE AND LIPID DROPLETS

INTRODUCTION

Free fatty acids are an essential source of energy for tissues. The flux of fatty acids is dependent on the lipolysis of stored triglycerides (TG) in the fat body. This organ is the principal site for storage of both glycogen and lipids, and plays a fundamental role in energy metabolism. The fat body fulfills many of the functions that in vertebrates are carried out by both the liver and adipose tissue (Law and Wells, 1989). Most of the information about lipid mobilization in insects comes from studies carried out in *Locusta migratoria* and *Manduca sexta*. In particular the adult (moth) *M. sexta* rely entirely on lipids as fuel for flight. Lipids are stored in the fat body adipocytes as cytoplasmic lipid droplets (Bailey et al., 1975; Willott et al., 1988). Lipid reserves in *M.sexta* are built up during the larval stages (Fernando-Warnakulasuriya et al, 1988) and utilized to sustain the life of the adult insect. The fat body mobilizes TG as sn-1,2-diacylglycerol (DG), which is released into the hemolymph (Tietz et al., 1975; Arrese and Wells, 1997) and loaded into the hemolymph lipoprotein, lipophorin. Lipophorin transports DG to the sites of utilization such as the flight muscle (van Heusden and Law, 1989), and ovaries

(van Antwerpen et al. 1998), where it is hydrolyzed to fatty acids by a lipophorin-lipase (van Antwerpen and Law, 1992).

Lipolysis is regulated by the neuropeptide adipokinetic hormone (AKH) (Gäde and Auesrswald, 2003). In the adult *M. sexta,* AKH stimulates the mobilization of stored TG, whereas in the larval stages the same hormone induces the mobilization of glycogen (Ziegler et al., 1990). Agents that raise intracellular cAMP concentration such as 8Br-cAMP or forskolin, also stimulate lipolysis in the adult insect fat body (Arrese et al., 1999). Moreover, AKH rapidly activates the fat body cAMP-dependent protein kinase (PKA) (Arrese et al., 1999). Therefore, AKH seems to promote an increase in lipolysis by activation of adenylyl cyclase and subsequent activation of cAMP activated kinase, PKA. AKH also induces a sustained increase in calcium influx in the fat body cells (Arrese et al., 1999, Lum and Chino, 1990; Wang et al., 1990), and agents that increase intracellular calcium concentration strongly stimulate lipolysis (Arrese et al., 1999).

In order to identify the target proteins involved in the mechanism of activation of lipolysis induced by AKH we investigated the changes on protein phosphorylation on two components of the lipolytic pathway: the TG-lipase - the enzyme responsible for mediating TG hydrolysis- and the lipid droplets -the organelles containing the TG substrate-. AKH induces a rapid phosphorylation of a lipid-droplet associated protein, Lsdp1 - lipid storage droplet protein 1-. The level of phosphorylation of Lsdp1 correlates with the activity of purified TG-lipase hydrolyzing TG contained in the lipid droplets. Furthermore, *in vitro* phosphorylation of the lipid droplets confirmed that PKA catalyzes the

phosphorylation of Lsdp1 (Patel et al., 2005). On the other hand, the fat body TG-lipase from adult *M. sexta* is a phosphorylatable protein of 76 kDa localized in the cytosol (Arrese and Wells, 1994). Given the role of PKA in activation of lipolysis, and the analogy between lipolysis in insects and vertebrates, it has been assumed that phosphorylation of the lipase would be involved in activation of lipolysis. However, recent studies showed that *in vitro* phosphorylation of TG-lipase mediated by PKA does not activate the enzyme (Patel et al, 2004, 2005). This observation raised the question of whether or not AKH actually promotes a change in the state of phosphorylation of TG-lipase *in vivo*. In the present study we performed *in vivo* phosphorylation experiments to monitor AKH-induced changes on TG-lipase phosphorylation. These studies along with a study of the lipase activity determined using an *in vitro* system containing *in vivo* radiolabeled TG-[^{3}H] lipid droplets, as substrate, and the cytosolic fraction of the fat body as source of enzyme led to the following conclusions: a) AKH does not change the state of phosphorylation of TG-lipase ruling out a direct effect of lipase phosphorylation on the activation of lipolysis; b) AKH induces changes in some other, unidentified, cytosolic factor/s which promote a moderate TG-lipase activation; c) It was confirmed that AKH-induced changes in the phosphorylation of the lipid droplet represents a major factor in the activation of the lipolytic cascade.

EXPERIMENTAL PROCEDURES

Materials: [^{32}P]-Orthophosphate was purchased from MP Biochemicals (Irvine, CA). Labeled trioleoylglycerol ([tri-9,10-^{3}H(N)]oleoylglycerol) and [9,10-(n)^{3}H]palmitic acid were purchased from PerkinElmer Life Sciences (Boston, MA). Protease and phosphatase inhibitors were purchased from Sigma (St Louis, MO). Q-Sepharose was from Amersham Biosciences (Piscataway, NJ). M.sexta AKH was obtained from Peninsula Laboratories (Belmont, CA). Electrophoresis items were from Invitrogen (Carlsbad, CA). Silica gel G plates were purchased from J.T. Baker (Phillipsburg, NJ). All of the other chemicals were of analytical grade.

Experimental Insects: *Manduca sexta* eggs were purchased from Carolina Biological supplies (NC), and larvae were reared on artificial diet (Bell and Joachim, 1976). Adult insects were kept at 25°C without food, and decapitated 24 h prior to being used. Before use, insects were injected with 13 mg of trehalose (Arrese et al., 1996b).

***In vivo* phosphorylation:** Experimental insects were injected with 250 μCi of [^{32}P] orthophosphate and 90 min later with 100 pmol of AKH. Fat body tissue was dissected 20 min after the hormonal injection. The tissue from four insects was pooled and homogenized with a Potter-Elvehjem glass homogenizer fitted with Teflon pestle, using 3 ml per fat body of homogenization buffer (Buffer H: 20 mM Tris, pH 7.4, 0.25 M sucrose, 1 mM EDTA, 0.1 mM benzamidine, 10 mg/l

leupeptine, 1 mg/l aprotonin, 0.1 % 2-mercaptoethanol, 2 mM imidazole, 2 mM sodium fluoride, 1.5 mM sodium molybdate, 1 mM sodium orthovanadate and 4 mM sodium potassium tartrate). The homogenate was layered with 2 ml of buffer H without sucrose and centrifuged at 100,000 x g for 1 hr. Tissue homogenates were fractionated into three fractions: cytosol (infranatant), membranes (pellet), and lipid droplets (fat cake).

The fat cake was resuspended in buffer H and gently vortexed. The sucrose concentration was adjusted to 15% (w/v) and a layer of 2 ml buffer H without sucrose was laid on top. Samples were centrifuged in a SW 40 rotor at 100,000 X g for 1 h. Purified lipid droplets were collected from the top. Typically lipid droplets of two insect fat bodies were resuspended in 0.5 ml of buffer H.

The pellet was resuspended in buffer H without sucrose and re-centrifuged at 100,000 x g for 1 h. The resulting pellet was dissolved in 1 ml of same buffer and centrifuged at 500 x g for 15 min. The resulting supernatant was used as membranes fraction.

The infranatant (cytosolic fraction) was passed through Q-Sepharose column equilibrated with buffer (10 mM Na_2HPO_4, pH-7.4, 1 mM EDTA, 0.1% 2-mercaptoethanol (v/v), 0.1 mM benzamidine 0.02% Triton-x-100, 10 mg/l leupeptine, 1 mg/l aprotonin, 2 mM imidazole, 2 mM sodium fluoride, 1.5 mM sodium molybdate, 1 mM sodium orthovanadate and 4 mM sodium potassium tartrate). After extensive wash with equilibration buffer, proteins were eluted with a NaCl gradient (20 mM-150 mM) in the same buffer. TG-lipase was eluted with

185 mM NaCl in the same buffer. The fractions containing TG-lipase activity were pooled, dialyzed, and concentrated.

Assay of TG-lipase activity: Lipase activity was assayed using micellar TG substrate as described previously (Arrese at al., 1994). The final assay volume of 0.1 ml contained 50 mM Tris, pH 7.9, 500 mM NaCl, 0.02 % (w/v) defatted bovine serum albumin, 0.5 mM EDTA, 2 mM dithiothreitol , 0.22 mM triolein [9,10-^{3}H, 0.043 µCi] and 1 mM Triton X-100. The reaction was initiated by adding enzyme. The mixture was gently vortexed for 20 s and incubated at 37 ºC with constant shaking. After 30 min, the reaction was terminated by the addition of 500 µl of an extraction mixture consisting of chloroform/ methanol/ benzene (2:2.4:1), containing 0.1 µmol of unlabeled oleic acid as carrier. Then, 40 µl of 1 N NaOH. The mixture was vortexed for 1 min and centrifuged at 2000 x g for 2 min. Aliquots of 150 µl from upper aqueous phase were transferred to scintillation vials for counting. Enzyme activity was expressed as nmol of TG hydrolyzed / min mg protein.

Polyacrylamide gel electrophoresis, autoradiography and densitometry analysis: SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (Laemmli, 1970). For the separation of lipid droplets-associated proteins, the concentration of SDS in the sample buffer was increased to 6% (w/v). Proteins were visualized by Coommassie Blue. The gel was dried and phosphorylation was visualized by autoradiography. Gels and

autoradiograms were scanned on an imaging densitometer (Bio-Rad model GS-700). The intensity of protein band and phosphorylation were quantified using the Multi analyst Macintosh software from the gel and autoradiogram scans, respectively. The intensity of phosphorylation was calculated as the ratio of the phosphorylation to the mass. The mass of TG-lipase was quantified by immunoblotting using antibody raised against the fat body TG-lipase. Nitrocellulose membranes were exposed on X-ray films and the intensity of the phosphorylation of TG-lipase band was measured by densitometry. The level of phosphorylation of TG-lipase was estimated as the ratio of the intensity of phosphorylation and the mass.

Western blot detection of TG-lipase: A polyclonal antibody against purified TG-lipase was raised in chicken at Cocalico Biologicals (Reamstown, PA). For western blotting, proteins were separated by SDS-PAGE (10%), transferred to nitrocellulose membrane and immunodetection was performed using anti-TG-lipase antibodies (1:200). After incubation of membranes with horseradish peroxidase-conjugated rabbit anti-chicken secondary antibody (1:50,000), peroxidase activity was detected using ECL chemiluminescence reagents (Amersham). X-ray films were scanned and the intensity of the positive signal was quantified by densitometry.

Preparation of endogenously radiolabeled [^{3}H]TG lipid droplets: Fat body lipids were radiolabeled during the fifth larval instar (Arrese and Wells, 1997).

Each insect was fed with 200 μCi of [9,10 (n)-^{3}H]palmitic acid. Fat body tissue from adult insects 2-3 days old was dissected 20 minutes after hormonal injection and lipid droplets were isolated. Lipid droplet samples were analyzed for protein and TG concentration, and radioactivity associated to TG. Lipid analysis of lipid droplet TG showed that 99.8 % of the radiolabel was localized in the fatty acyl residues. sn-(1)3 positions of TG contained 89 ± 2% of the label. Analysis of the distribution of radioactivity among neutral lipid class of the lipid droplets showed that TG contained 97 ± 0.7 % radioactivity.

TG-lipase activity hydrolyzing TG from [^{3}H]TG-lipid droplets: An aliquot of the lipid droplet preparation containing 100 nmol of TG was transferred to a glass tube containing lipase reaction buffer. The reaction was initiated by adding an aliquot of cytosol containing 7.5 μg of total protein in a final volume of 150 μl. Final reaction conditions were 50 mM Tris, pH 7.9, 500 mM NaCl, 0.02 % (w/v) bovine serum albumin, 0.67 mM TG, 0.37 mM Triton X-100, and 2 mM dithiothretiol. The mixture was gently vortexed for 20 s and incubated at 37 °C with constant shaking. After 30 min, the reaction was terminated by addition of 750 μl of chloroform: methanol (2:1) and 5 μl of 6 N HCl. The mixture was vortexed for 1 minute, and centrifuged at 2000 g for 2 minutes. The organic phase was collected, and counts/min was measured in an aliquot. The remaining solvent was dried under the stream of nitrogen, and the lipids were separated by thin-layer chromatography on silica gel G plates using hexane: ethyl ether: formic acid (70:30:3) as the developing solvent (Arrese and Wells, 1994). MG, DG, fatty

acid, and TG fractions were visualized by I_2 vapors and scraped from the plates. Radioactivity associated with each fraction was determined by liquid scintillation counting. Blank reactions in which the cytosol was omitted, but incubated at 37ºC parallel to reactions samples were used to obtain the basal level of distribution of radioactivity among lipid classes and calculate the percentage of hydrolysis. The lipase activity was expressed as nmol of TG hydrolyzed / min mg protein.

Protein and TG Content of Lipid Droplets: Lipid droplet associated proteins were precipitated with acetone (85% v/v) at -20 °C. Afterwards samples were centrifuged at 10,000 X g for 5 min and the pellet was dissolved in 10% SDS (w/v). An aliquot of 50 µl (~15 µg of total protein) was used to measure total protein using the BCA method in a final volume of 1ml (Smith et al, 1985).

TG concentration was determined using the Infinity Triglyceride reagent kit as described by manufacturer (ThermoTrace Ltd, Melbourne, Australia). Triolein was used as standard.

Statistics: Statistical comparisons were made by the Student's t test. $P < 0.05$ was considered to be significant.

RESULTS

Level of TG-lipase phosphorylation

Although TG-lipase can be phosphorylated *in vitro* by PKA purified from *M. sexta* fat body, the phosphorylation did not modify the enzymatic activity to hydrolyze TG contained either in an artificial substrate (micelles of Triton X-100) or the native substrate (lipid droplets) (Patel et al., 2004; Patel et al., 2005). This information raised the question whether or not the phosphorylation state of lipase changes upon hormonal stimulation. To address this issue we performed *in vivo* phosphorylation experiments by ^{32}P-radiolabeling the intracellular pool of ATP. Preliminary experiments showed that high and similar levels of protein phosphorylation were reached at 90 and 120 min after the injection of ^{32}P-orthophosphate into the hemolymph. Thus, for the rest of the experiments, insects were injected with ^{32}P -orthophosphate for 90 min prior to the injection of hormone. Hormonal stimulation was done for 20 min, and unstimulated tissue provided the level of protein phosphorylation corresponding to basal lipolysis. TG-lipase is localized in the cytosol and has an electrophoretic mobility which is very close to that of the storage proteins. Because the storage proteins are very abundant accounting for more than 85 % of the cytosolic proteins to study the phosphorylation of TG-lipase it was necessary to remove them. This was done by passing each cytosol (stimulated and basal) through an anionic exchange resin (Q Sepharose) as indicated under Methods. **Figure 18A** shows the TG-lipase activity of the fractions eluted from Q-Sepharose with 185 mM NaCl corresponding to the cytosol fractions obtained from insects control and treated

with AKH. A moderate (1.6 fold) increase of lipase activity was detected in the samples from stimulated tissue. The fractions containing the majority of lipase activity (32-34) were pooled, and subjected to SDS-PAGE. After transferring the proteins to nitrocellulose membranes, the mass and phosphorylation level of TG-lipase were determined by immunoblotting and autoradiography as indicated in Methods. As shown in Panel B (**Figure 18**), the western blots show that the majority of the lipase was contained in the peak fractions 32-34 (lanes 2). The western blot of fractions 25-31 and 35-40 are shown in lanes 1 and 3, respectively. It should be noted that the amount of total protein loaded in lanes 1 and 3 was twice (14 µg) the amount loaded in lane 2 (7µg). **Figure 18** panel C shows the autoradiography of the proteins in fractions 32-34. Similar levels of TG-lipase phosphorylation were observed in control and AKH-treated insects. TG-lipase was found to be constitutively phosphorylated and *in vivo* stimulation of lipolysis induced by AKH did not affect the level of phosphorylation of TG-lipase.

Lipid droplet-associated proteins were also separated by SDS-PAGE. Coomassie blue stained gels revealed about twenty different proteins ranging between 31 to 120 kDa. Confirming previous observations, the autoradiograms of ^{32}P-phosphoproteins of AKH lipid droplets showed an important change in the phosphorylation state of Lsdp1 a protein that migrates as a doublet of 42.6 / 44.2 kDa (**Figure 19**).

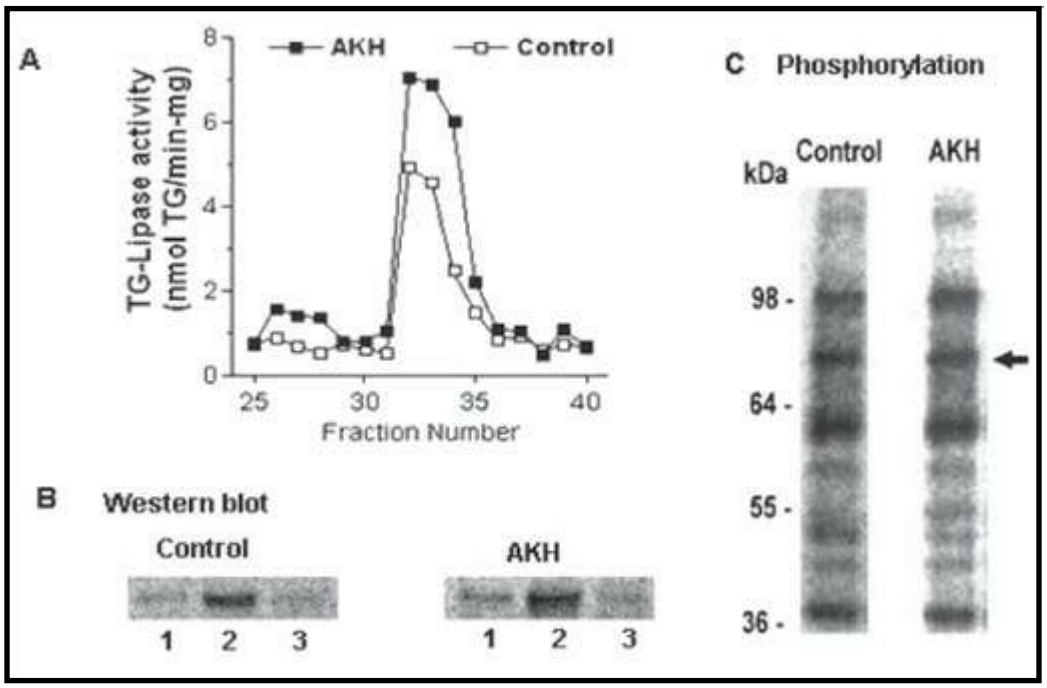


Figure 18. Panel A- Elution profile of TG-lipase from Q-Sepharose column: Adult *Manduca sexta* were injected with [$^{32}PO_4H_3$] and, after 90 min, with buffer (control, —□—) or AKH (—■—). After 20 min, the fat body tissue was isolated and homogenized. The cytosolic extract, 100,000 x g supernatant of homogenate, was loaded into the column. The column was developed using sodium chloride gradient in equilibration buffer. TG-lipase was eluted with 185 mM NaCl in the same buffer. Lipase activity was determined against an emulsion of [^{3}H]-triolein and Triton X-100 as indicated in Methods. Determinations were done in duplicate. Panel B- Western Blotting: 7 µg of protein from fractions 32 to 34 (lanes 2) and 14 µg from fractions 25 to 31 (lanes 1) and 35 to 40 (lanes 3) were separated by SDS-PAGE, transferred to nitrocellulose and analyzed by western blotting using anti-lipase antibody as indicated in Methods. Panel C-Phosphorylation of *M. sexta* fat body TG-lipase: *In vivo* ^{32}P radiolabeled proteins eluting in fractions 32 to 34 were pooled and separated on 8% SDS-PAGE, transferred to nitrocellulose followed by autoradiography. 7 µg of protein for the pool of fractions 32-34 was loaded in the gel. The position of TG-lipase is indicated by the arrow.

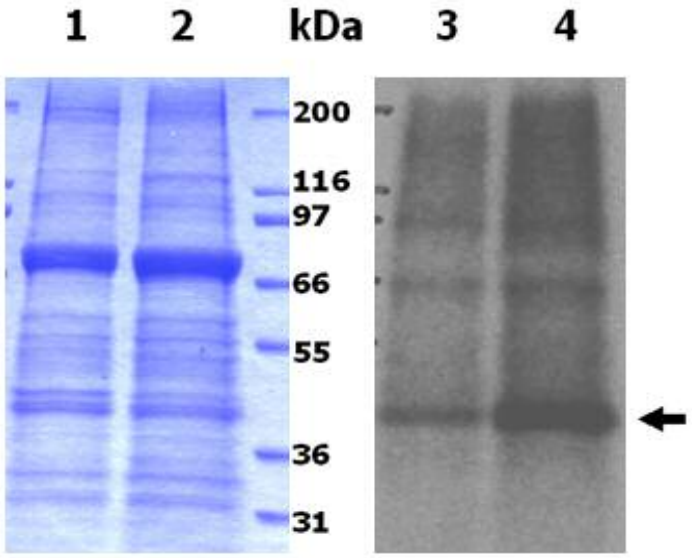


Figure 19. SDS-PAGE and Autoradiography of phosphorylated lipid droplet proteins: *In vivo* ^{32}P-radiolabeled lipid droplets proteins (10 µg) were resolved on 10% SDS-PAGE and stained with Coomassie Brilliant Blue followed by autoradiography. Lanes 1 and 3 show the gel and autoradiography, respectively, of control-lipid droplets; lanes 2 and 4 depict the gel and autoradiography of AKH- lipid droplets. The position of Lsdp1 is indicated by the arrow.

The comparison of the level of phosphorylation of TG-lipase and Lsdp1 showed that while the phosphorylation state of Lsdp1 increased 280%, the level of phosphorylation of TG-lipase increased 9 % and that difference was not statistically significant (*P*=0.31) (**Figure 20**). Comparisons of the level of phosphorylation among the proteins of the three subcellular fractions showed that the increase in the phosphorylation of lipid droplet associated protein Lsdp1 was the major change induced by AKH (data not shown).

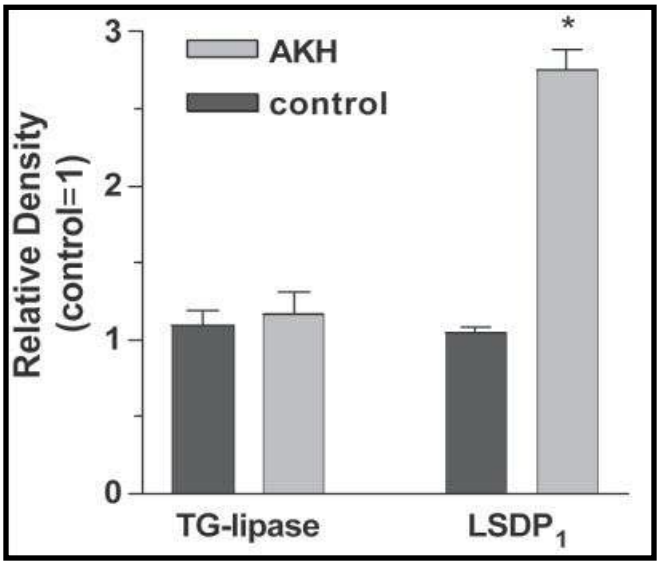


Figure 20. Level of phosphorylation of TG-lipase and $Lsdp_1$ from the fat body of control and AKH-stimulated insects**.** Phosphorylation was quantified by densitometry as the ratio of the intensity of phosphorylation (autoradiography) to the mass (western blot). Values are relative to control values. Data represent the mean ± S.E (n = 5). Asterisk denotes a significant difference versus the control

Contribution of AKH-induced changes in the cytosol and lipid droplets to the activation of lipolysis

Since AKH induced a moderate activation of TG-lipase from fat body cytosol and a significant activation of the lipid droplets; we decided to determine the overall effect of AKH on the lipolysis under *in vitro* conditions. For this purpose we determined the rates of TG-hydrolysis catalyzed by cytosols isolated from control and AKH-treated insects against *in vivo* radiolabeled [^{3}H]TG-lipid droplets isolated from the corresponding insects. The comparison of the rates of TG-hydrolysis (**Figure 21**, "a" and "d") shows a significant difference between control and AKH samples (P<0.05). The lipase activity of AKH-cytosol hydrolyzing AKH-lipid droplets (11.1 ± 2.1 nmol TG/ min-mg) was 3.1-fold higher than the lipase activity of control-cytosol hydrolyzing control-lipid droplets (3.6 ± 0.5 nmol TG/ min-mg).

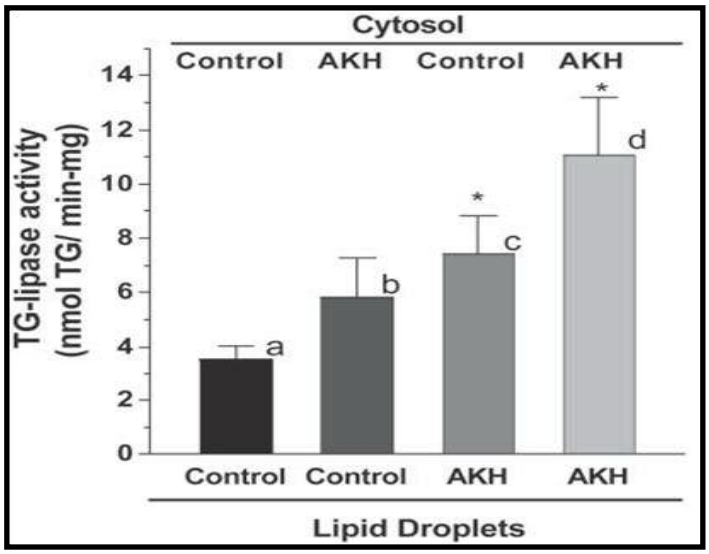


Figure 21. Effect of activation of the enzyme and substrate on the activity of TG lipase: [^{3}H]TG-lipid droplets and cytosolic fractions were isolated from basal (control) and AKH-stimulated (AKH) fat bodies. Aliquots of lipid droplets containing 100 nmol of TG were used to measure the TG-lipase activity of cytosols which is expressed in nmol TG hydrolyzed/ min mg protein. Data represent the mean ± S.E (n=4); P^{ab} =0.09, P^{cd} =0.09, P^{ac} =0.02, P^{bd} =0.04, and P^{ad}=0.006. The asterisks denote a significant difference versus the basal condition (control cytosol and lipid droplets).

In order to estimate the contributions from cytosol and lipid droplets to the overall process we also determined the lipase activity of control-cytosol against AKH-[^{3}H]TG-lipid droplets (**Figure 21** "c") and vice versa (**Figure 21** "b"). Comparison of control- and AKH-cytosol measured against control-lipid droplets ("a" and "b" in **Figure 21**) showed an increase in lipase activity from 3.6 ± 0.5 to 5.8 ± 1.5 nmol TG / min-mg (*P*=0.09). A similar effect of AKH was also observed when the lipase activities of the cytosols were measured against AKH-lipid droplets ("c" and "d"), the activity increased from 7.4 ±1.4 (control-cytosol) to 11.1 ± 2.1 nmol TG/ min-mg (AKH-cytosol), (*P*=0.09*).* The significance of the differences observed is low. However, the data still suggest that changes in the

cytosol could contribute to the overall lipolytic response provoked by AKH stimulation.

As shown in the **Figure 21** (“a” and “c”), the lipase activity of control-cytosol is significantly (P<0.05) lower hydrolyzing control-lipid droplets (“a” 3.6 ± 0.5 nmol TG/ min-mg) than AKH- lipid droplets (“c” 7.4 ± 1.4 nmol TG/ min-mg). A similar conclusion was reached when the activity of cytosol isolated from AKH treated insects was determined against control- and AKH-lipid droplets. The activity of AKH-cytosol was also significantly lower (P<0.05) when measured against control-lipid droplets (“b” 5.8 ± 1.4 nmolTG / min-mg) than against AKH-lipid droplets (“d” 11.1 ± 2.1 nmolTG / min-mg). These results indicate that the changes induced by AKH on the lipid droplets contribute with approximately a 2-fold increase to the overall rate of lipolysis.

DISCUSSION

Triglycerides are contained in the lipid droplets of fat body adipocytes. The lipid droplets are complex organelles composed of an outer layer of phospholipid and protein and an inner core containing more hydrophobic molecules such as TG (Londos et al. 1999: Brown, 2001). In order to hydrolyze TG, the TG-lipase must access the substrate and therefore it must interact with the lipo-protein surface of the lipid droplet. Given the location of the lipase substrate, the lipase activity is expected to be dependent on a number of physical chemical factors that define the properties of the lipid droplet surface. Among these factors, the surface concentration of the lipase substrate, the phospholipid composition and charge of the surface, as well as on the interaction with other proteins that cover the lipid droplet surface could play key roles on the lipase activity. Thus, in order to study the biochemical factors involved in the activation of the lipase, it is important to use *in vitro* systems that resemble as much as possible the physiological conditions. We have recently described the use of *in vivo* TG-radiolabeled lipid droplets to determine the TG-lipase activity and used this approach to study the activity of purified TG-Lipase (Patel et al., 2005). In the present study we extended the use of radiolabeled lipid droplet to assess the role of cytosol and lipid droplets in the activation of lipolysis.

The results obtained with this *in vitro* system indicate that AKH promotes a ~3.1-fold increase in the rate of lipolysis. This study clearly showed that AKH-induced modifications in the lipid droplets play a major role in activation of the lipolysis. Independently of the metabolic condition of the insect, the lipolytic

activity of cytosols showed a 2-fold increase (2.1-fold with control cytosol and 1.9-fold with AKH cytosol) with lipid droplets from AKH treated insects. A comparison with the overall increase in lipolysis indicates that AKH-induced changes in the lipid droplets accounts for ~70% of the lipolytic response elicited by AKH. As recently reported (Patel et al., 2005), and also observed in the present study, activation of the lipid droplets appears to be directly related to phosphorylation of Lsdp-1.

On the other hand, comparison of the TG-lipase activities of cytosols suggested that AKH evokes some changes in the cytosol. As described in Results, the lipase activity of AKH-cytosol was 1.6-fold (against control-lipid droplets) or 1.5-fold (against AKH-lipid droplets) greater than the activity of control cytosol. Although the significance of these individual increases is lower, the fact that similar values were obtained with control and AKH lipid droplets gives further significance to the activation of the cytosol. Furthermore, the activation of the cytosol would be supported by the fact that activation of the lipid droplets, which represent a 2-fold increase, can only account for 70% of the overall activation process (3.1-fold increase in lipolysis). The ~1.5-fold increase in the cytosol activity of AKH treated insects would account for the remaining 30%.

Since AKH induces a rapid activation of fat body PKA (Arrese et al., 1999) as well as a moderate activation of TG-lipase (Arrese et al, 1996, Patel et al., 2004), it was thought that, in analogy to the human sensitive lipase (Strålfors and Belfrage, 1983: Olsson et al., 1984), fat body lipase activation was induced by PKA catalyzed phosphorylation (Arrese et al., 1999). Because TG-lipase can

be phosphorylated *in vitro* by purified PKA from the insect fat body, but the phosphorylation of the lipase failed to increase the enzymatic activity (Patel et al., 2004; Patel et al., 2005), we investigated whether or not AKH induces the phosphorylation of the TG-lipase. This study showed that the fat body TG-lipase from *Manduca sexta* is phosphorylated under basal conditions, and unlike Lsdp1, its level of phosphorylation remains unchanged upon stimulation of lipolysis by AKH. Therefore the increase of lipase activity of the cytosols induced by AKH is independent of the phosphorylation state of this protein. This confirms our previous suggestion based on results using the purified lipase that other proteins could be involved in the activation of lipase (Patel et al., 2004). The contribution of the cytosol to the lipolytic response could arise from a factor other than the lipase. For instance, AKH could trigger changes in the association of the lipase with other protein partners and, thus, modify the accessibility of the enzyme to the substrate or its activity.

The protein partners could be typical cytosolic protein or proteins detached from the lipid droplet (e.g. Lsdp1). The elucidation of this issue will be a matter of future studies.

CHAPTER V

THE MAIN TRIGLYCERIDE-LIPASE FROM THE INSECT FAT BODY IS AN ACTIVE PHOSPHOLIPASE A_1: IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF THE PHOSPHOLIPASE ACTIVITY

INTRODUCTION

The fat body is the principal organ for storage of lipids in insects. Triglycerides (TG) constitute the main lipid form representing about 90% of the total fat body lipids (Bailey, 1975; Arrese and Wells, 1997). The content of TG in the fat body is influenced by several factors, including development stage, nutritional state, sex, migratory flight. In the tobacco hornworm, *Manduca sexta*, which is widely used as a model insect, the content of fat body TG increases continuously until the end of the larval (feeding) period from few micrograms to ~ 80 mg (Fernando-Warnakulasuriya et al., 1988). During subsequent development, lipid reserves are mobilized to sustain the life of the adult insect (moth), which feeds occasionally (Arrese et al., 2001; Ziegler, 1991). TG is stored in fat body adipocytes as lipid droplets within the cytoplasm (Willot et al., 1988). Unlike vertebrates, where stored fatty acids are mobilized as free fatty acids, most fatty acids are released from the fat body to hemolymph as *sn*-1,2-diacylglycerol (DG) (Arrese and Wells, 1997; Arrese et al., 1996). In circulation DG is carried by lipophorin, the insect lipoprotein, for delivery to tissues, *e.g.* the

flight muscle, and ovaries, where it is hydrolyzed to fatty acids by a membrane-bound lipophorin-lipase (Van Antwerpen and Law, 1992).

TG lipolysis is under hormonal regulation by the neuropeptide adipokinetic hormone (AKH) (Gade and Auerswald, 2003), which elicits a glucagon-like action mediated by G protein-coupled receptor that activates both inositol phosphate and cAMP signaling responses (Gade et al., 1997; Staubli et al., 2002). In *M. sexta* the effect of AKH on the mobilization of energy reserves is dependent on the developmental stage. During the larval stage AKH mobilizes glycogen through the activation of glycogen-phosphorylase whereas it promotes a massive lipolytic response in the adult stage (Ziegler et al., 1990).The lipolytic response induced by AKH is associated with a rapid activation of fat body cAMP-dependent protein kinase A (PKA) and a sustained increase in calcium influx (Arrese et al., 1999). The mobilization of TG is mediated by lipolytic enzymes. The major TG-lipase of the fat body has been purified from adult *M.sexta* (Arrese and Wells, 1994). This is a cytosolic enzyme with a molecular mass of 76 kDa that can be phosphorylated *in vitro* by PKA. Contrary to expectations, phosphorylation of the lipase induces only minor changes in the lipase activity (Patel et al., 2004; Patel et al., 2005). TG-lipase is constitutively phosphorylated and the level of phosphorylation remains constant when lipolysis is stimulated (Patel et al., 2006). Recent *in vivo* and *in vitro* experiments have shown that the enzyme does not bind tightly to the lipid droplets and its activity is highly correlated with the phosphorylation level of a lipid droplet-associated protein, Lsdp1 (Patel et al., 2005). AKH-induced lipolysis provokes a rapid

phosphorylation of Lsdp1, a protein that shares a small region of sequence identity with perilipin A from mammalian lipid droplets (Miura et al., 2002), and this event accounts for the majority of the lipolytic response induced by AKH (Patel et al., 2005 and 2006). Although significant details of the mechanism of lipolysis are emerging, these studies and many studies carried out in adipocytes of vertebrates clearly show that activation of lipolysis is a complex process that involves cytosolic proteins and lipid droplets associated proteins (Marcinkiewicz et al., 2006). The details of the interactions and signals that ultimately lead to an increase in the rate of TG hydrolysis remain to be elucidated. Here, we report the identification of the TG-lipase from *M. sexta* as the homolog of CG8552 (FlyBase annotation) from the fruit fly, *Drosophila melanogaster*. This study shows that the enzyme is conserved among insects and shares significant sequence similarity with vertebrate phospholipases from PA-PAL$_1$. The identification of the lipase prompted us to study its possible phospholipase activity. Our data demonstrate that fat body TG-lipase has phospholipase A_1 activity, which allows the hydrolysis of the phospholipid monolayer of the lipid droplets. This finding suggests that the phospholipase activity of the insect lipase is sufficient to allow access of the lipase to TG which for the most part is contained in the core of the lipid droplets.

EXPERIMENTAL PROCEDURES

Materials: [^{32}P]-Orthophosphate was purchased from MP Biochemicals (Irvine, CA). L-α-1-palmitoyl-2-[1-^{14}C]-oleoyl-sn-glycerol-3-phospho-choline was purchased from American Radiolabeled Chemicals (St.Louis, MO). [tri-9,10-^{3}H(N)]oleoylglycerol was purchased from PerkinElmer Life Sciences (Boston, MA). DEAE Sepharose Fast Flow, Phenyl Sepharose, Q Sepharose, and Ni Sepharose High Performance were purchased from Amersham Biosciences (Piscataway, NJ). Hydroxyapatite Bio-Gel HT Gel was from Bio-Rad (CA). *M.sexta* AKH was obtained from Peninsula Laboratories (Belmont CA). BEL, DEDA and MAFP were purchased from Alexis Biochemicals (San Diego, CA). Protease and phosphatase inhibitors were purchased from Sigma-Aldrich. Silica gel K6 plates were purchased from Whatman (Maidstone, England). pIEx-1 Ek/LIC vector, *Escherichia coli* strain NovaBlue, BL21(DE3), Insect Gene Juice were obtained from Novagen (Madison, WI). Total mRNA from adult *Drosophila melanogaster*, *Taq* HiFi polymerase, dNTPs, Sf9 cells and Sf-900 II SFM medium were purchased from Invitrogen Corporation (Carlsbad, CA). DNA sequencing was performed by the Department of Biochemistry (Oklahoma State University) Core Facility using an ABI Model 3700 DNA Analyzer. All other chemicals were of analytical grade.

Experimental Insects: *M. sexta* eggs were purchased from Carolina Biological supplies, and larvae were reared on artificial diet (Bell and Joachim, 1976). Adult insects were maintained at room temperature without food. To achieve consistent

basal levels of lipolysis, the insects were decapitated 24 hours ahead of the experiment and injected with 13 mg of trehalose two hours before the experiments (Arrese et al., 1996).

Purification of TG-lipase: TG-lipase was purified from the cytosolic fraction of *M. sexta* fat body homogenates as reported previously with minor modifications (Arrese and Wells, 1994). Fat body tissue from 200 insects was collected in ice cold homogenization buffer (buffer H: 20 mM Tris, pH 7.4, 0.25 M sucrose, 1 mM EDTA, 0.1 mM benzamidine, 10 mg/l leupeptin, 1 mg/l aprotonin, 0.1 % 2-mercaptoethanol). The tissue was homogenized with a Potter-Elvehjem glass homogenizer fitted with Teflon pestle and centrifuged at 100,000 x g for 1 hr. The cytosolic fraction was used to purify TG-lipase using a combination of anion-exchange (DEAE Sepharose and Q Sepharose), hydroxyapatite and hydrophobic interaction (Phenyl Sepharose) chromatography.

Protein Identification by Mass Spectrometry: Purified TG-lipase was separated by SDS-PAGE on 8% gel. The gel was stained with Coomassie Blue and the band was excised from the gel. The protein was cleaved with trypsin and then sequenced by microcapillary reverse-phase high pressure liquid chromatography nanoelectrospray tandem mass spectrometry on a Finnigan LCQ DECA XP Plus quadrupole ion trap mass spectrophotometer at Harvard Microchemistry Facility. The MS/MS spectra were correlated with known algorithm Sequest and program developed in that laboratory (Chittum et al.,

1998). Protein sequence analysis by Edman degradation of some peptides separated by HPLC after trypsin digestion was also performed at the same facility.

Expression and partial purification of recombinant CG8552: Total mRNA from adult *Drosophila melanogaster* was reversed transcribed using oligod $(T)_{18}$-primer. The resulting cDNA was used to amplify the coding region of CG8552 (Accesion number NP_609185) from position 4203 -corresponding to the second methionine, amino acid residue 1354- to the C term -amino acid residue 2016- by PCR. The primers were 5′-GACGACGACAAGATGGCAAGTGCCAGCAGCGAAAGGGCCA-3′ and 5′-GAGGAGAAGCCCGGTTCACATCGGCAACAGCGAGTGGCTAAC-3′. The 5′ end of the primers incorporated the ligation-independent cloning (LIC) sequences (underlined). The amplified product was ligated into the vector pIEx-1 Ek/LIC after being treated with LIC-qualified T4 DNA polymerase. pIEx vector contains N-terminal His-Tag and S-Tag coding sequences and is designed for transient transfection and protein expression in Sf9 cells. The generated plasmid (pIEx-CG8552) was then transformed into *E. coli* strain NovaBlue, and the positive clones were confirmed by DNA sequencing. Sf9 (*Spodoptera frugiperda*) cells that were cultured in Sf9-900 II SFM medium were transfected with pIEx-CG8552 dissolved in Insect Gene Juice (Novagen, EMD Biosciences) according to manufacturer's instruction. Suspension cultures were grown at 28°C with shaking at 140 rpm. Cells contained in 30 ml culture were harvested 48-h post-transfection and sedimented by centrifugation. Cells were resuspended in 5 volumes of cold homogenization buffer (20 mM phosphate, pH

7.8, 20% glycerol, 5mM DTT, 1 mM EDTA, aprotinin 1 mg/l) and lysed by sonication on ice in two steps of 30 sec each. The crude lysate was centrifuged first at 300 *g*, 20 min and then at 100,000 x *g* (1 h, 4°C) to separate cytosolic and membrane fractions. The 100,00 x *g* pellet contained most of the recombinant protein and was used for the purification. The pellet that was resuspended in a volume of homogenization buffer equivalent to the volume of cytosol fraction was combined with an equal volume of a solution containing 20 mM Tris pH 7.9, 500 mM NaCl, 2 M urea and incubated for 2 h at 4 °C with orbital shaking. The solution was adjusted to 40 mM imidazole and combined with 2 ml of resin pre-equilibrated with the same buffer. The slurry was incubated for 4 h. The resin was washed with ten bed volumes of wash buffer I (20 mM Tris, 500 mM NaCl, 40 mM imidazole,0.5 mM DTT, pH7.9), followed by five bed volumes of wash buffer II (20 mM Tris, 500 mM NaCl, 75 mM imidazole,0.5 mM DTT, pH7.9). The flow was stopped, and the column was incubated for 4 h with one volume of elution buffer (20 mM Tris, 500 mM NaCl, 75 mM imidazole, 50 mM EDTA, 0.5 mM DTT, pH7.9). Fractions were assayed for TG-lipase activity as described below.

Assay for TG-lipase Activity: The final assay volume of 0.1 ml contained 50 mM Tris, pH 7.9, 500 mM NaCl, 0.02 % (w/v) defatted bovine serum albumin (BSA), 0.5 mM EDTA, 2 mM dithiothreitol (DTT), 0.44 mM [9,10-^{3}H] triolein (1.9mci / mmol) and 2 mM Triton X-100 and purified TG-lipase (0.5µg). The mixture was incubated at 37 ºC with constant shaking. After 30 min, the reaction was terminated by the addition of 500 µl of a mixture of chloroform-methanol-

benzene (2/2.4/1, v/v/v) and 40 µl NaOH 1N. Aliquots of 150 µl from upper aqueous phase were transferred to scintillation vials for counting. Blank reactions did not contain enzyme. Enzyme activity was expressed as nmol of FFA /min-mg.

Assays for TG-lipase activity of recombinant protein was performed under the same conditions as indicated above using [9,10-^{3}H] triolein (7.6 mci / mmol) as substrate. Reaction was stopped by addition of 500 µl of chloroform-methanol (2:1, v/v) and 40 µl HCl 1 N. Radiolabeled lipids from the organic phase were separated on TLC using hexanes:ethyl ether:formic acid (70:30:3 v/v/v) as developing solvent. Regions of plate corresponding to TG, DG, MG and FFA were scrapped and quantified by liquid scintillation counting.

Assay of Phospholipase activity: The final assay volume of 0.1 ml contained 50 mM Tris, pH 7.9, 500 mM NaCl, 0.02 % (w/v) defatted BSA, 0.5 mM EDTA, 2 mM DTT, 2 nmol of 1-palmitoyl-2-[1-^{14}C]oleoyl-sn-glycerol-3- phosphocholine (18 mCi/ mmol) and 2 mM Triton X-100 and purified TG-lipase (0.5µg). The mixture was incubated at 37 ºC with constant shaking. After 30 min, the reaction was terminated by the addition of 500 µl of chloroform/ methanol (2:1) and 5 µl of 6 N HCl. The organic phase was collected and dried. The lipids were separated by thin-layer chromatography (TLC) on Silica Gel K6 plates using chloroform/methanol/water (50:25:4) as the developing solvent. Spots corresponding to PC, LPC (phospholipase A_1 activity), and FFA (phospholipase A_2 activity) were scraped and their radioactivity determined by liquid scintillation counting. Blank reactions did not contain enzyme. Enzyme activity was

expressed as nmol of LPC and FFA / min-mg. The activity against phosphatidic acid (PA) was assayed under the same experimental conditions. PA was prepared by incubating 1-palmitoyl-2-[1-^{14}C]oleoyl-sn-glycerol-3- phosphocholine with phospholipase D (type IV from cabbage). PA was purified by TLC and eluted from silica using a mixture of chloroform/ methanol/ acetic acid/ water (50/39/1/10). In inhibition studies the purified enzyme was pre-incubated for 10 min at room temperature with inhibitor prior to the measurement of activity

Preparation of endogenously [^{32}P]-phospholipid-labeled lipid droplets:

Insects were injected with 200 µCi of [^{32}P]-orthophosphate. After 36 hr, insects were decapitated and injected with trehalose as indicated above. Tissue from two insects was pooled and homogenized in 6 ml of buffer H. Lipid droplets were purified as previously described (Patel et al., 2005). Typically, lipid droplets of 2 insect fat bodies were resuspended in 0.5 ml of buffer. For lipid droplets under high lipolysis conditions, lipolysis was stimulated by injection of 100 pmol of AKH, whereas injection of buffer provided the basal lipolysis. The adipokinetic effect was confirmed by the phosphorylation of Lsdp1- the major phosphoprotein of the lipid droplet whose phosphorylarion is induced by AKH- that was monitored by SDS-PAGE and autoradiography (data not shown).

Lipid composition of lipid droplets: Total lipids were extracted by adding 5 volumes of chloroform-methanol (v/v) (Folch et al., 1957). The lipids in the extracts were separated by TLC using hexane-ethyl ether-formic acid 70/30/3

(v/v/v) as the developing solvent (Arrese et al., 2001). Plates were sprayed with 3% cupric acetate in 8% orthophosphoric acid (v/v) and heating at 200ºC for charring (Hamilton and Hamilton, 1992). Plates were scanned on an imaging densitometer (Bio-Rad model GS-700). The intensity of spots was quantified using the Multi Analyst Macintosh software. Results were expressed as percentage of total lipids (PL, MG, DG, TG, FFA, cholesterol, and cholesterol ester). Neutral glycerides were determined in the lipid extracts using the Infinity triglyceride reagent kit as described by the manufacturer (ThermoTrace Ltd, Melbourne, Australia). Phospholipids were determined by measuring inorganic phosphorous after digestion in deionized water and perchloric acid for 1 h at 180 °C followed by addition of ammonium molybdate and ascorbic acid (Rouser et al., 1970). The sample was further heated for 5 min in a boiling water bath and cooled, and the absorbance read at 800 nm to quantify total phosphorous. The mole ratio of neutral glycerides to PL was 102 ±9.4:1.

Polar lipids from lipid droplet lipids extract were separated by TLC using chloroform-methanol-ammonia 28% (65/25/5) or chloroform-methanol-acetic acid-water (50/30/8/4) as the developing solvent. Individual phospholipids of the lipid droplets were made visible by charring as described above. Phospholipids were identified by comparison with standards run on the same plates. When analyzing [^{32}P]-phospholipid-labeled lipid droplets, ^{32}P-phospholipid forms were visualized by autoradiography. The intensity of phospholipid spots from the plate and autoradiogram scans were quantified as indicated above. Results were expressed as percentage of total phospholipids or total radioactivity, respectively.

Phospholipase and TG-lipase activity against [^{32}P]-labeled lipid droplets: An aliquot of lipid droplet preparation containing 1 nmol PL and 100 nmol of TG was transferred to a glass tube containing TG-lipase reaction buffer. The reaction was initiated by adding TG-lipase (3 µg) in a final volume of 150 µl. Final reaction conditions were 50 mM Tris, pH 7.9, 500 mM NaCl, 0.02% (w/v) BSA, 2 mM DTT, 0.60 mM TG and 0.17 mM Triton X-100 (Patel et al., 2005). The mixture was gently vortexed for 20 s and incubated at 37 ºC with constant shaking. After 30-45 min, the reaction was terminated by the addition of 750 µl of chloroform/methanol (2/1) and 5 µl of 6 N HCl. The organic phase was collected and dried. The lipids were separated by TLC using chloroform/methanol/ ammonia (65/25/5) as the developing solvent. ^{32}P-labeled phospholipids were visualized by autoradiography. Autoradiograms were scanned and analyzed as described above. Blank reactions in which TG-lipase was omitted were used to obtain basal level of distribution of radioactivity. For TG-lipase activity, the spots corresponding to FFA (visualized by I_2 vapors) were scraped from the plate, and fatty acids were eluted from silica using chloroform. The amount of FFA was estimated using non-esterified fatty acid detection kit as described by manufacturer (Wako Chemicals USA, Inc).

Other methods: Protein concentrations were determined by the Bradford dye-binding assay (Bradford, 1976) using bovine serum albumin as standard. SDS-PAGE was performed according to Laemmli (Laemmli, 1970) and proteins were visualized by Coomassie Brilliant Blue R staining. Two different anti-lipase

antibodies were generated in chickens at Cocalico Biologicals (Reamstown, PA). The first antibody was produced using purified *Manduca sexta* TG-lipase as antigen. A second antibody was obtained using a mixture of two peptides (ERPVSHESSVSHSL and VGRVEVLPISWHGHLHSEE) that were coupled to KLH. Anti-S.Tag monoclonal antibody was purchased from Novagen (Madison, WI). Immunodetection was performed using the corresponding horseradish peroxidase-conjugated secondary antibody and ECL chemiluminescence reagents (Amersham Biosciences).

Statistical comparisons were made by the Student's t test. $p \leq 0.05$ was considered to be significant.

RESULTS

Identification of fat body TG-lipase

To identify the triglyceride-lipase from the insect fat body, TG-lipase from *M. sexta* was purified to homogeneity following a previously reported procedure (Arrese and Wells, 1994), in which the enzyme activity is monitored using [^{3}H]-triolein as substrate. The final preparation showed a single band in a 2-D PAGE with a relative mass of 74-76kDa and a pI of 5.8-6.0. The protein band obtained after the separation by SDS-PAGE on 8% acrylamide was excised and subjected to identification by LC/MS/MS as indicated in Material and Methods. LC/MS/MS revealed the presence of three peptides sequences that matched to *D. melanogaster* (Dme) CG8552-PA: CSWFYK, SVEEVVDDFR, MHLELK. **Figure 22** shows the region of CG8552-PA (Accesion number NP_609185) in

which these peptides are found. This region is 662 amino acids long and extends from the second methionine (amino acid residue 1354) to the C term (amino acid residue 2016). In addition, internal sequence of *M.sexta* TG-lipase was obtained by Edman degradation. The following peptide sequences were obtained: VEVLPIS, ATSLQLVQSHYK, YHWFYSVDVEDK. The sequence VEVLPIS is found in CG8552-PA, whereas the other two peptides shared a high degree of identity with two regions also present in CG8552-PA (**Figure 22**).

Partial transcripts from *Anopheles gambiae* (Aga, Accession number XP_312576) and *Apis mellifera* (Ama, Accession number XP_392149) similar to CG8552 have been reported. Both translates also contain similar sequences to those found in *M. sexta* TG-lipase (**Figure 22**). The complete coding sequence of CG8552 from Dme encodes for a product of 2016- amino acid residues. The alignment of the deduced amino acid sequences from different insects shows a region of high conservation (53-58% identity) localized between amino acid 1354 to 2016 of the Dme product (**Figure 22**). This region includes the lipase consensus sequence (GXSXG), containing the active site Ser essential for catalysis (29) (**Figure 22**). In addition, two conserved domains, a DDHD domain (Accession number: PF02862) and a WWE domain (Accession number: PF02825), are also found in the same region. The DDHD is a long domain (180 residues) towards the C terminus named after these four residues that may form a metal binding site (**Figure 22**). The WWE domain that is named after three of its conserved residues is localized between amino acid residues 1368 and 1446 of the Drosophila protein (**Figure 22**). The WWE domain has been identified in

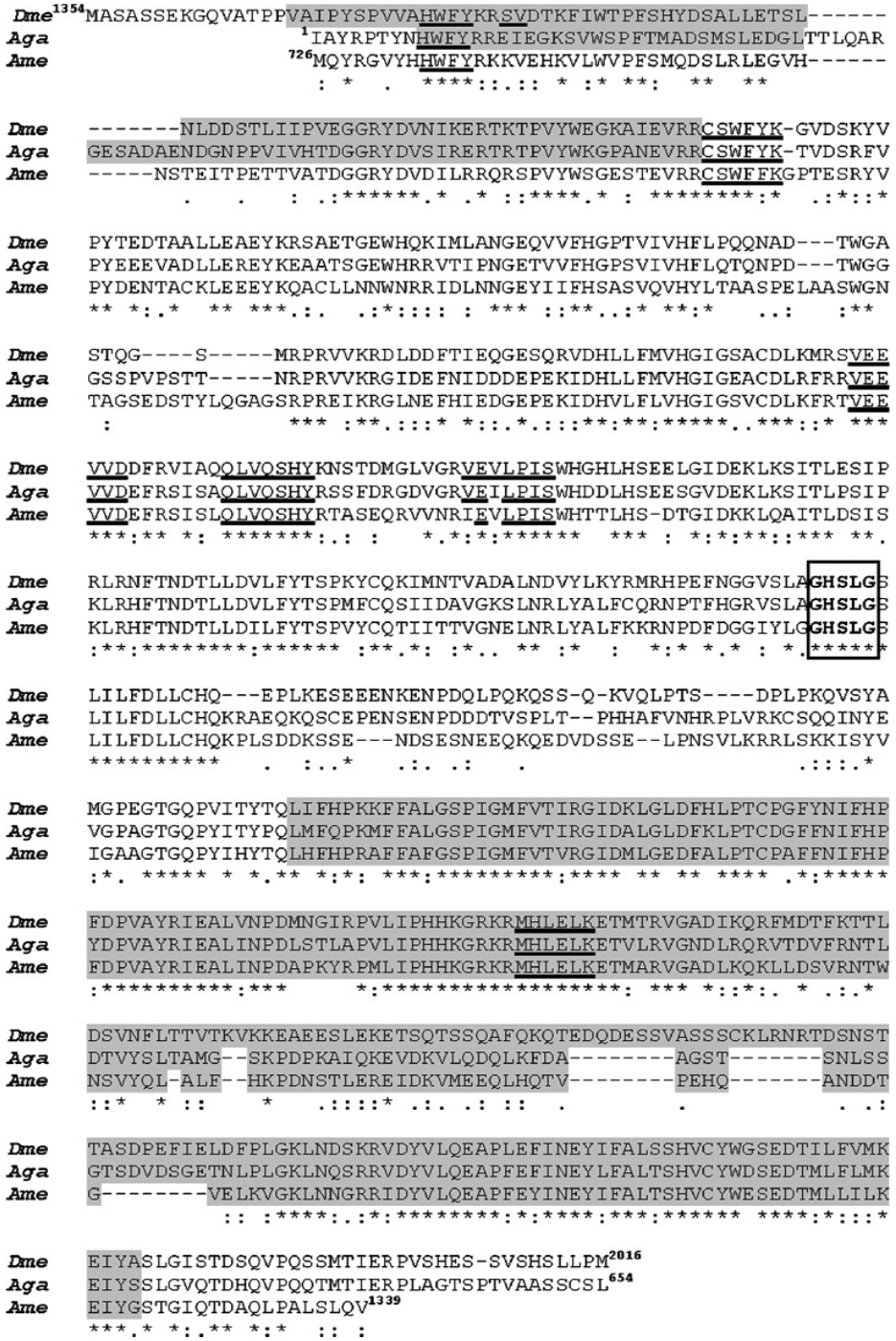


Figure 22. Amino acid sequence alignment of the partial deduced sequence from transcript CG8552 from *Drosophila melanogaster* (*Dme*, fruit fly) and corresponding homologues from *Anopheles gambiae* (*Aga*, mosquito) and *Apis mellifera* (*Ame*, honey bee). The amino acid microsequence obtained from *Manduca sexta* is underlined. The numbering on the left indicates the position of that amino acid in the deduced amino acid sequence. Lines in the sequence are gaps introduced by the program (ClustalW) to optimize the alignment. Identical residues in all the sequences are denoted by asterisks; conservative substitutions are denoted by dots. The putative lipase catalytic site is boxed. Conserved domains are boxed in gray.

diverse proteins with predicted ubiquitin- and ADP-ribosylation-related functions, and it is predicted to mediate specific protein-protein interactions (Aravind, 2001).

In order to confirm the identification of the *Manduca sexta* TG-lipase as the homolog of CG 8552 from the fruit fly, the region localized between the second methionine (amino acid 1354) to the C-term (aminoacid 2016) of the Dme product shown in Figure 22 was cloned and expressed in the insect cell line Sf9 as indicated in Material and Methods. The protein was expressed as a fusion protein containing a His_{10}-Tag and S-Tag at the N-Terminal with an estimated size of 80.6 kDa that was confirmed by Western blot using S-Tag antibody. A single band of the predicted size was displayed in homogenates of transfected cells and was absent in the corresponding control cells (**Figure 23A**). In addition Western blots using two antibodies that react with *Manduca sexta* lipase were also performed. The first anti-lipase antibody was generated against purified *M.sexta* TG-lipase whereas the second anti-lipase antibody was produced using a mixture of two peptides found in CG8552-PA sequence from Dme. **Figure 23B** shows the Western blots of purified *Manduca sexta* TG-lipase (76 kDa) and recombinant CG8552 (80.6 kDa) probed with different antibodies. The antibody against *M.sexta* TG-lipase recognized the recombinant protein from CG8552 (**Figure 23B**-panel 1). On the other hand the antibody against the peptides recognized *M.sexta* TG-lipase (**Figure 23B**-panel 2). As expected the antibody against the S-tag only recognized the fusion protein and did not react with purified *M.sexta* TG-lipase (**Figure 23B**-panel 3). The immuno cross-reactivities of the proteins and the presence of the lipase consensus sequence in CG8552-

PA are consistent with the identification of *M.sexta* fat body TG-lipase based on the MS/MS study and the internal peptide sequences obtained by Edman degradation.

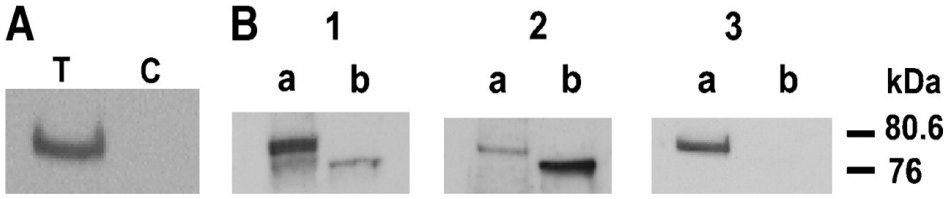


Figure 23. Western analysis of recombinant Dme CG8552-(His_{10}-S.Tag). A**)** Homogenates of transfected (T) Sf9 cells (30 µg/lane) and control (C) cells (30 µg/lane) were separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-S.Tag antibody mouse monoclonal. Immunoreactive bands were visualized by probing the blot with an anti-mouse IgG horseradish peroxidase conjugate followed by reaction with ECL reagents and exposure to film. B**)** Blots containing purified *Manduca sexta* TG-lipase (lanes a, 1 µg/lane) and expressing Sf9 cells homogenates (lanes b, 30 µg/lane) were analyzed by Western blot using three different antibodies. 1-Anti-*Manduca sexta* lipase; 2- Anti-peptides-CG8552; 3- Anti-S.Tag antibody.

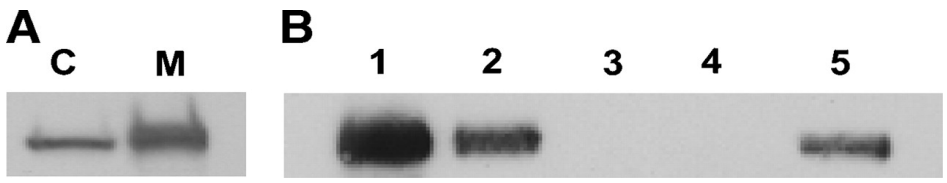


Figure 24. Western analysis of the subcellular distribution and Ni affinity purification of recombinant Dme CG8552-(His_{10}-S.Tag). A) Cytosol (C) and membrane (M) proteins expressing Sf9 cells were separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-S-Tag. To normalize the load, 0.002 volume of each sample was loaded in each well. B**)** Membrane fraction resuspended in homogenization buffer was incubated in a solution containing 1M urea, 40 mM imidazole, 250 mM NaCl, 0.5 mM DTT, pH 7.9 with Ni Sepharose. Resin was loaded in a column and developed as indicated in Materials and Methods. Samples from all chromatographic steps were analyzed by Western blot using anti-S.Tag antibody. 1-sample loaded in the resin (30 µg); 2-flow through (30 µg); 3- 40mM imidazole (2 µg); 4- 75 mM imidazole (2 µg) ;5- 50 mM EDTA elution in the presence of 75 mM imidazole (4 µg).

For further characterization the recombinant protein was affinity purified and its ability to hydrolyze triglyceride determined. The majority (82.1± 5.7%) of the recombinant CG8552 protein was associated with the membrane fraction

(**Figure 24A**). The pellet was incubated in the presence of 1 M urea as described in Materials and Methods. The mixture was adjusted to 40 mM imidazole and incubated with Ni Sepharose resin. Under these conditions most of the fusion protein (87± 13%) bound to the resin. After extensive washes with 40 and 75 mM imidazole, the fusion protein was eluted with 50 mM EDTA in the presence of 75 mM imidazole. Elution of the fusion protein can be done with 200 mM imidazole. However imidazole was avoided because at high concentration inhibited *M.sexta* TG-lipase activity. The fractions were analyzed by Western blot and TG-lipase activity. The fraction eluted with 50 mM EDTA contained recombinant protein and also exhibited TG-lipase activity (130 ± 4 nmol TG hydrolyzed/ min-mg) (**Figure 24B**).

A Blast search revealed two proteins with high sequence similarity in two regions of CG8552: a phospholipase A1 (KIAA0725p) and p125, a Sec23-interacting protein. CG8552 showed 40.9% and 34.3% identity in a 696 aa and 1000 aa overlap with KIAA0725 and p125, respectively. Likewise a region of CG8552 (321 aa) containing the lipase conserved sequence exhibits 30.5% identity with the phosphatidic acid-preferring phospholipase A1 (PA-PLA_1, Accession number Q8NEL9) (not shown). As a note the lipase consensus sequence in PA-PLA_1 has a serine residue instead of glycine (SHSLG) (Higgs et al., 1998). In mammals these three proteins (KIAA0725p, p125, and PA-PLA_1) are being referred as the PA-PLA_1 family (Tina et al., 1999).

Phospholipase and TG-lipase activities co-purifiy and are inhibited or resistant to identical lipase inhibitors

Preliminary experiments with *M.sexta* purified lipase indicated that the enzyme had phospholipase activity. In order to confirm that this activity was inherent to the TG-lipase rather than to a contaminant, fat body TG-lipase

Table 4. TG-lipase and phospholipase activity during the purification of TG-lipase from *M. sexta* fat body

	TG-Lipase nmol FFA /min-mg	**Phospholipase**		**Ratio TG/PL hydrolyzed**
		nmol LPC /min-mg	nmol FFA /min-mg	
Cytosol	0.27± 0.04	0.007± 0.001	0.012 ± 0.001	14
DEAE I	8.5 ± 0.4	0.04 ± 0.004	0.064 ± 0.003	82
DEAE II	17.2 ± 0.6	0.08 ± 0.001	0.098 ± 0.005	97
Phenyl Sepharose I	38.0 ± 1.8	0.13 ± 0.002	0.18 ± 0.01	123
Hydroxyapatite	565 ± 4	1.00 ± 0.01	0.58 ± 0.06	357
Q Sepharose	730 ±18	1.50 ± 0.02	0.80 ± 0.04	317
Phenyl Sepharose II	1086± 8	2.21± 0.07	0.89 ± 0.11	350

Lipase activities hydrolyzing [^{3}H]-triolein and 1-palmitoyl-2-[1-^{14}C]-oleoyl-sn-glycerol-3-phosphocholine were determined after each purification step as described in Material and Methods. Data represent the mean ± S.E. (n=3).

was purified monitoring the lipase activity against [^{3}H]-triolein and determining phospholipase activity of the fractions containing TG-lipase activity. **Table 4** shows TG-lipase and PL-lipase activities during the purification. The preparations showed a significantly higher activity hydrolyzing TG than PL, and both specific activities were increasing with the purification. The final preparation represented a 4000–fold purification of TG-lipase, and showed a major protein band in a SDS-PAGE of molecular mass 76kDa (**Figure 25A**). Western blotting analysis

confirmed that the band corresponds to TG-lipase (**Figure 25B**). The proportion of TG versus PL hydrolyzed was used to assess the progress of the lipase purification. This ratio increased during the first steps of the purification indicating the presence of other phospholipases. However, the ratio remained relatively constant through the last three chromatographic steps. At the same time the degree of purification of the TG-lipase was increasing as judged by the specific activity and the protein composition observed in the SDS-PAGE gel. Altogether the data indicated that the phospholipase activity associated with the TG-lipase is an intrinsic activity of the enzyme. Moreover, Ni-affinity purified recombinant CG8552 protein was also assayed for phospholipase activity. The recombinant protein hydrolyzed [^{14}C]-PC at a rate of 0.35± 0.01 nmol PC / min-mg. This result provides further support to the finding indicating that *M.sexta* TG-lipase also possesses phospholipase activity.

The specificity of the phospholipase activity of the insect lipase was determined using 1-palmitoyl-2[1-^{14}C]oleoyl-sn-glycerol-3-phosphocholine solubilized in Triton X-100 micelles. TLC analysis of the radiolabeled products of PL hydrolysis showed that 70 % was [^{14}C]-lysoPC and 30 % [^{14}C]-fatty acid indicating a lipase preference for the sn-1 position. Moreover, as previously shown for the TG-lipase activity, the phospholipase activity was calcium independent.

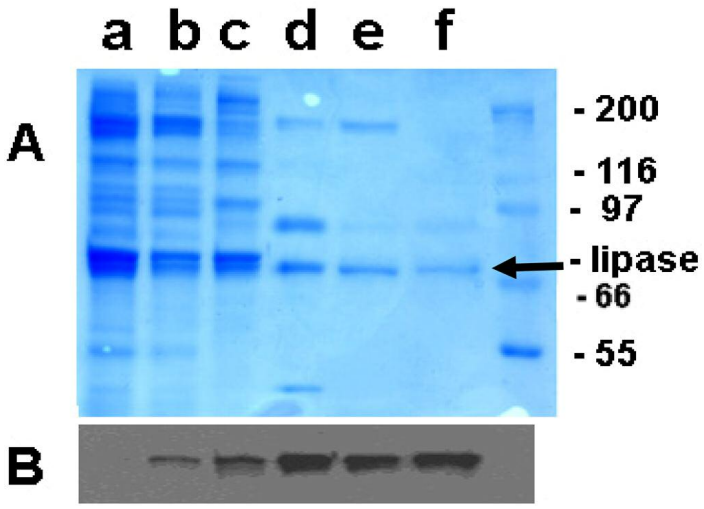


Figure 25. *M. sexta* fat body TG-lipase purification. A**)** SDS-PAGE: a) and b) first and second DEAE (20 µg and 10 µg, respectively); c) first Phenyl Sepharose (8µg); d) hydroxyapatite (4.5µg); e)Q Sepharose (2.5 µg); f) second Phenyl Sepharose (1µg); B**)** Western Blot of the same fractions. 10 µg of protein was loaded in lanes a), b) and c); 2 µg in lane d), and 1.4 µg and 1 µg in lanes e) and f), respectively. The faint band at ~92 kDa seen in lane f was identified by maldi-tof as glycogen phosphorylase.

Given the homology found between *M. sexta* lipase and vertebrate phospholipases from PA-PAL$_1$ family, it was interesting to know whether the *M. sexta* TG-lipase hydrolyzes phosphatidic acid (PA). The enzyme hydrolyzed PA at a lower rate than PC under the same assay conditions, 1.07± 0.04 nmol PA /min-mg and 2.85 ± 0.04 nmol PC/ min-mg, respectively. Likewise, TG-lipase showed a strong preference for sn-1 position of PA.

Phospholipases inhibitors were evaluated for their abilities to discriminate between the PL- and TG-lipase activities. DEDA (7,7-dimethyleicosadienoic acid) and BEL (bromoenol-lactone), which are PLA$_2$ inhibitors, were unable to inhibit PL- or TG-lipase activities at the expected concentrations. On the other hand, the phospholipase activity was sensitive to MAFP, a methyl arachidonyl flurophosphonate analog of arachidonic acid, that binds irreversibley to serine residues and is a potent phospholipase inhibitor (IC$_{50}$= 0.5-5 µM) (Lio et al.,

1996). Preincubation of the enzyme with 5μM MAFP inhibited both lipase activities to a similar extent (83.5 ± 8.2 % inhibition of PLase and 84.5 ± 2.0 % of TG-lipase) providing further support to the notion that a single active site is involved in the hydrolysis of PL and TG.

The concentration-dependence of lipase activity with TG and PL substrates determined in micellar substrates of Triton X-100 micelles at pH 7.9, 0.5 M NaCl, 0.5 mM EDTA, and 2 mM DTT showed saturation kinetics with apparent K_ms of 152 ± 11 μM and 7.8 ± 1.1 μM, respectively, and Vmax of 560 ± 10 nmol TG/ min-mg and 5.5 ± 0.3 nmol PC/min-mg, respectively. These values indicate that the enzyme has a strong (5-fold) preference for acylglycerides over phospholipids.

Phospholipase activity against the native susbtrate (lipid droplets)

The fact that TG-lipase has phospholipase activity against the micellar substrate suggested that the enzyme may catalyze the hydrolysis of native phospholipids present in the surface of the lipid droplets. As shown in **Table 5**, lipids of lipid droplets isolated from fat tissue of *M. sexta* consist of TG with small amounts of PL, DG, FFA and cholesterol ester. This lipid composition is consistent with lipid compositions of lipid droplets isolated from other sources (Okuda et al., 1994; Zweytick et al., 2000). PC and PE were the main phospholipid forms representing 60% of total phospholipids (**Table 5**). Although most lipid droplets isolated from other systems also exhibited PC as the major phospholipids

component, a higher content of PE and lysoPL was observed in *M.sexta* lipid droplets than in lipid droplets from vertebrate adipose tissue or plants.

Table 5. Lipid composition of the lipid droplet isolated from *M.sexta* fat body

Total Lipids (%)		Phospholipids (%)	
TG	87.8 ± 0.4	**PC**	41.2 ± 1.1
1,2-DG	3.5 ± 0.4	**PE**	21.0 ± 0.6
FFA	3.4 ± 0.2	**LPC**	12.0 ± 1.0
CE	3.2 ± 0.3	**LPE**	9.1 ± 1.0
CHO	0.6 ± 0.2	**PS+PI**	12.0 ± 2.1
PL	0.8 ± 0.1	**SM**	2.5 ± 0.5

Values of total lipid composition were expressed as percentage of total lipids (PL, phospholipids; CHO, cholesterol; CE, cholesterol ester; FFA, fatty acids; DG, diglyceride; TG, triglyceride). Values of phospholipid composition were expressed as percentage of total phospholipids (PC, phosphatydilcholine; PE, phosphatydilethanolamine; LPC, lysoPC; LPE, lysoPE; PS, phosphatydilserine; PI, phosphatydil-inositol; SM, shpingomyelin). Data represent the mean ± S.E. (n=3).

In order to assay phospholipase activity of TG-lipase against the lipid droplets, cellular phospholipids were radiolabeled *in vivo* and lipid droplets were isolated. The distribution of radioactivity among radiolabeled phospholipids of lipid droplets was: PC and PE represented the 39.8 ± 1.9 and 20.2± 0.3% of total radioactive phospholipids, whereas radioactive LPC and LPE were radiolabeled 14.9 ± 0.5 and 9.6 ± 1.2 %, respectively. PS and PI combined represented 14.6 ± 0.14 %, and SM was 3.0 ± 0.3% of total radioactive phospholipids. The comparison of the distribution of radioactivity among phospholipids was very close to the distribution of mass (**Table 5**) indicating that the radiolabeling procedure yielded homogenously radiolabeled phospholipids. [^{32}P]-lipid droplets were incubated with purified TG-lipase under the conditions previously identified to measure TG-lipase activity against the native substrate (Patel et al., 2005).

After reaction, the main PL classes were separated by TLC and quantified by autoradiography. The decrease of radioactivity associated with PC and PE with the concomitant increase of radioactivity in LPC and LPE proved that the enzyme has the ability to hydrolyze both PC and PE (**Figure 26 A-B**).

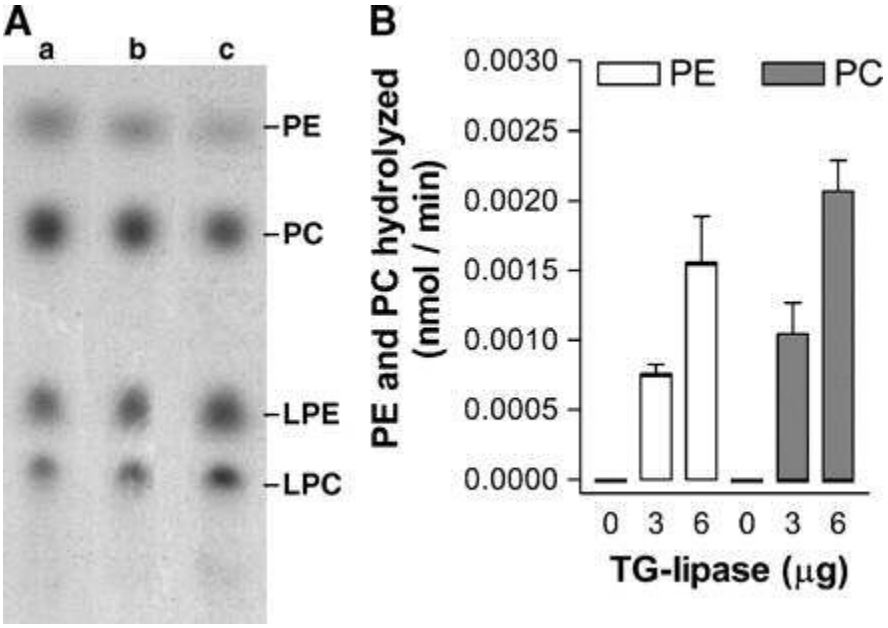


Figure 26. Phospholipase activity of TG-lipase against endogenously [^{32}P-phospholipids]-labeled lipid droplets. A**)** TG-lipase was incubated with [^{32}P]PL-radiolabeled lipid droplets that were isolated from insects with basal lipolytic activity. PL classes were separated by TLC and quantified by autoradiography and densitometry. The figure shows the autoradiogram of a representative TLC plate obtained with: (a) control lipid droplets (no lipase); (b) lipid droplets incubated with 3μg of purified TG lipase; (c) lipid droplets incubated with 6 μg of purified TG-lipase. All reactions contained 1nmol of total phospholipids (100 nmol of TG) and were incubated for 45 min. PE, phosphatidylethanolamine; PC, phosphatidylcholine; LPE, lysoPE and LPC, lysoPC. B) Phospholipase activity expressed as nmol of PC and PE hydrolyzed / min by 3 μg and 6 μg of TG-lipase. To calculate the amount of PC and PE hydrolyzed, the values of the blank (incubation of the substrate with 0 μg TG-lipase) were substracted to the corresponding amount of PC and PE. Data represent the mean ± S.E. (n=4).

A simultaneous analysis of the TG-lipase activity hydrolyzing PL and TG contained in the lipid droplets showed that the enzyme hydrolyzes 77 molecules of TG for every molecule of PL contained in the lipid droplets (Table 3). The ratio

of hydrolysis of TG to PL determined with lipid droplets was much lower than the corresponding ratio obtained with separate micellar substrates under similar conditions. Using the Km and Vmax values obtained with micelles, and the concentrations of TG and PL found in the lipid droplets one would expect a ratio of activities of 750. The fact that the ratio of hydrolysis of TG to PL determined with lipid droplets is much lower than the ratio estimated from the data obtained with micelles is indicative of the lower accessibility of TG in the lipid droplets. On the other hand, consistent with a surface location of PC in the lipid droplets, similar phospholipase activities were observed in micelles and lipid droplets (**Table 6**).

Table 6. Lipase activity of TG-lipase against native and artificial substrates

Substrate	**Assay conditions** [a]	**Lipase activity** (nmol/min-mg)	
		TG	**PL**
Lipid droplets	600μM TG, 2.8μM PC, 1.4 μM PE, 0.17 mM Triton X-100,	77.2 ± 1.2	1.07 ± 0.05
[^{3}H]-Triolein in micelles	660μM TG, 2.7 mM Triton X-100	455 ± 12	
[^{14}C]-PC in micelles	4μM PC, 0.68 mM Triton X-100		1.63 ± 0.02

TG- and PL-lipase activities of the enzyme were determined with the native substrate (lipid droplets) and the artificial substrate (micelles of Triton X-100) as described in Material and Methods under the conditions specified in the table. Data represent the mean ± S.E. (n=3).
[a] All reactions were performed in 50 mM Tris, 500 mM NaCl, 0.5 mM EDTA, 2 mM DTT, pH 7.9.

In order to evaluate a possible role of the phospholipase activity *in vivo*, the phospholipid compositions of the lipid droplets isolated from control and lipolyticaly stimulated insects (5, 10, 20 and 30 min after hormonal injection) were analyzed. This study showed that the relative content of PE and PC slightly

decreased in the lipid droplets isolated from stimulated tissue. Significant differences in the content of the major phospholipids of the lipid droplets were found 10 and 20 min after the hormonal stimulation (**Figure 27A**). The reduction of PL content was accompanied by a bigger reduction in the content of lipid droplets TG (**Figure 27B**).

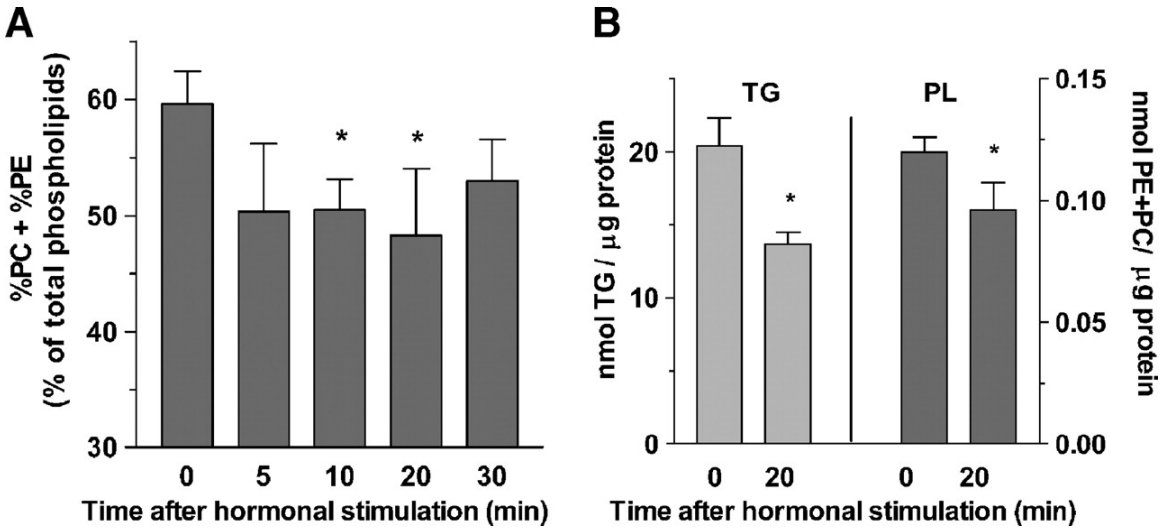


Figure 27. Effect of stimulation of lipolysis on the content of the major phospholipids of the lipid droplets**.** A) [^{32}P]PL-radiolabeled lipid droplets were isolated from insects with basal and stimulated (5, 10, 20 and 30 min after AKH treatment) lipolysis and analyzed by TLC and autoradiography. The intensity of phospholipid spots from autoradiogram scans was quantified as indicated in Material and Methods. Values were expressed as percentage of total phospholipids. B) The content of TG and PE+PC of the lipid droplets is expressed as nmol / µg of total lipid droplet proten. Data represent the mean ± S.E. (n=4-8). *, $p \leq 0.05$ vs control.

DISCUSSION

Lipolysis of TG is extremely active during long-term flight in insects such as *M. sexta*, and this process can be mimicked by the injection of AKH in the hemolymph (Arrese et al., 1996). Mobilization of TG stores from lipid droplets is catalyzed by TG-lipases. The fat body of *M.sexta* has a major cytosolic TG-lipase that represents the only insect TG-lipase purified and characterized (Arrese and Wells, 1994). This study shows that TG-lipase is the homolog of *D. melanogaster* CG8552. Based on homology searches, the predicted functions for this gene are phospholipase A_1 activity and metal ion binding. This work shows that a major function of CG8552 is the hydrolysis of TG in insect adipocytes and confirms that, as predicted, the enzyme has phospholipase activity. Furthermore, this gene appears to be conserved among insects (**Figure 22**).

CG8552 has a 6.5 kb transcript that encodes for a 214 kDa putative protein. This translation product is larger than the *M. sexta* TG-lipase. However, analysis of the coding sequence using GeneMark.SPL identified the presence of alternative start codons. Although the consensus sequence for Drosophila (Cavener and Krasney, 1991) is not present at neither of these start codons, according to Kozak (Kozak, 2005) the sequence context in the proximity of the second start codon (at position 4203) seems more favorable for initiation of translation than the first start codon. This raises the possibility that the functional initiaton site could be located at the second start codon. Initiation of translation at the second start codon would result in a 662 amino acids long protein with a calculated molecular mass of 75kDa and a theoretical isoelectric point of 5.62.

These calculated parameters coincide with the experimental values obtained with the purified TG-lipase. All the peptide sequences obtained from *M. sexta* as well as the sequence GHSLG identified as the active site corresponding to the consensus sequence GXSXG characteristic for lipases are present in that region of 662 amino acid depicted in **Figure 22**. The fact that the recombinant protein generated from the second initiation codon of CG8552 to the stop codon that was expressed in Sf9 cells showed both TG- and PL- lipase activites supports this possibility. Alternatively, initiation of translation at the first start codon would imply that the lipase is the result of post-translational modification of a much larger precursor. In that case an unusual protein of ~139kDa that lacks internal methionine residues would be also produced in addition to TG-lipase.

Comparison between the insect lipase and other proteins

Drosophila CG8552 has significant sequence similarities with the proteins p125, KIAA0725, and PA-PAL$_1$. These proteins share a central and C-terminal region and were considered to form the PA-PLA$_1$ mammalian family (Tani et al., 1999; Nakajima et al., 2002). KIAA0725 is a cytosolic phospholipase A$_1$ ubiquitously expressed in human tissues whose physiological function is unknown (Nakajima et al., 2002) whereas PA-PLA$_1$ is a cytosolic phospholipase highly expressed in human testis that preferentially hydrolyzes phosphatidic acid (Higgs et al., 1998). The expression of these enzymes in adipose tissue is unknown. p125 localizes in endoplasmic reticulum (ER), and has high sequence similarity with KIAA0725. It also contains an N-terminal proline-rich region responsible for the interaction

with Sec23p (Tani et al., 1999), and seems to be involved in the organization of ER exit sites (Shimoi et al., 2005).

At least three distinct regions can be recognized in Drosophila CG8552, the lipase consensus sequence, and the conserved domains WWE and DDHD. KIAA0725 is the only member of PA-PLA$_1$ mammalian family that has all the regions present in CG8552. Moreover, PA-PLA$_1$ possesses a coil-coil forming region and a potential transmembrane domain (Higgs et al., 1998). p125, KIAA00725, and CG8552 also exhibit a potential transmembrane region that is localized in a region that contains the lipase active site. On the other hand, there is no indication of potential coil-coil region in CG8552 nor KIA00725 as it is seen in PA-PLA$_1$ and p125. Altogether the information suggests a closer functional relationship between CG8552 and KIAA00725 than between CG8552 and the other two proteins.

Genes of the PA-PLA$_1$ family were found in yeast (YORO22c), C. elegans (MO3A1.6), and plants (SGR2) (Kato et al., 2002). These genes encode proteins that share less similarity with TG-lipase. The proteins of this family have in common a sequence containing a lipase active site and a DDHD domain. However, phospholipase activity is exhibited by PA-PLA$_1$ (Higgs et al., 1994), KIAA00725 (Nakajima et al., 2002) and CG8552 (this study) but not by p125 (Nakajima et al., 2002) and SGR2 (Kato et al., 2002). The biological function of proteins of PA-PAL$_1$ family is unknown or not well defined. The present study identifies CG8552 as a major player in the hydrolysis of TG in insects. This

information could be beneficial for the characterization of other proteins of this family.

The lipase identified in this study is unrelated to the known TG-lipases identified so far from vertebrate adipocytes. The fat body TG-lipase shares several functional similarities with HSL (Stralfors et al., 1987) such as its preference for the primary ester bonds of TG, faster hydrolysis of DG than TG (2- and 10-fold for TG-lipase and HSL, respectively), phosphorylation by PKA, and reducing conditions for activity. On the other hand, HSL has no detectable phospholipase activity (Yeaman, 2004), and it does not have significant sequence similarity. In addition to the extensively studied HSL, four new TG-lipases from vertebrate adipocytes have been recently disclosed (Jenkins et al., 2004; Soni et al., 2004; Zimmermann et al., 2004). Interestingly, three of them, adiponutrin, TTS-2.2, and GS2, are members of the calcium-independent phospholipase A_2 family. These proteins have a high TG lipase activity and acyl-transacylase activity and a much lower phospholipase activity (Jenkins et al., 2004). TTS-2.2, also designated desnutrin or ATGL, might act coordinately in the catabolism of TG (Zimmermann et al., 2004) particularly under basal conditions (Langin et al., 2005). An ATGL homolog in Drosophila has been reported, and a functional role of this lipase on lipid homeostasis in Drosophila has been indicated, since the loss of ATGL activity causes obese flies whereas its overexpression depletes the fat stores (Gronke et al., 2005). The insect TG-lipase identified in this study is unrelated to the ATGL homolog or any other

protein from Drosophila including the previously reported lipases lip1, lip2 and lip3 that have been identified through homology searches (Pistillo et al., 1998).

Lipoprotein lipase (LPL), hepatic lipase (HL), pancreatic lipase (PL) and endothelial lipase (EL) are enzymes of the lipase gene family that hydrolyze triglycerides and phospholipids to different extents (Hide et al., 1992; McCoy et al., 2002). The ratio of TG-lipase to PL-lipase activity for the human lipases is 833, 139.9, 24.1 and 0.65 for PL, LPL, HL and EL, respectively (McCoy et al., 2002; Verger et al., 1984). This ratio for *M.sexta* fat body TG-lipase activities measured with the artificial and natural substrates was 350 and 72, respectively. Within the limitation of this comparison due to the difficulties of comparing lipase activity determined under different conditions, these ratios indicate that the lipase activity *M. sexta* TG-lipase on phospholipids is intermediate between LPL and PL.

Role of the Phospholipase Activity in the Activity of TG-lipase

The action of the lipase on TG contained in the lipid droplets requires its intimate association with the lipid substrate. All types of lipid droplets identified so far are characterized by a hydrophobic core coated by a monolayer of phospholipids embedded with proteins (Zweytick et al., 2000). The process by which the lipase gains access to TG is unknown. Given the low solubility of TG in both water and phospholipids, the surface of the lipids droplets is likely to have a very low concentration of TG. Experimental estimation of the solubility of TG in PL bilayers and monolayers indicate that between 1 and 2 % of TG could be solubilized in

phospholipids surface (Spooner et al., 1987; Handa et al., 1992). A comparison of the kinetic data obtained in this study also suggests that the surface concentration of TG in the lipid droplet is very low. This low surface TG concentration is expected to limit the accessibility of the lipase to the TG substrate and thus the rate of lipolysis. However, in this context, one could envisage that the intrinsic phospholipase activity of the lipase could generate a higher local concentration of TG resulting in a higher rate of lipolysis. As shown in this study, the fat body TG-lipase was capable of hydrolyzing the main phospholipids of the insect lipid droplets, PC and PE, *in vitro.* On the other hand, analysis of the phospholipid composition of the lipid droplets isolated from insects under basal and stimulated lipolysis revealed that the lipolytic process stimulated by AKH involves partial hydrolysis of main phospholipids of the lipid droplets. Altogether these results support the notion that the dual phospholipase/TG lipase activity of the lipase could actually take place *in vivo*. Based on this information it is proposed that the phospholipase activity of the insect TG-lipase is sufficient to allow access of the lipase to TG molecules contained in the core of the lipid particles. The fact that several TG-lipases from vertebrate adipocytes that have TG lipase activity also are capable of hydrolyzing phospholipids supports the idea that the dual lipase activity could be a requirement of lipases hydrolyzing TG in lipid droplets. On the other hand, TG-lipases lacking phospholipase activity, such as HSL, could require additional steps of activation to allow access of enzyme to the core of the lipid droplets as it has been previously speculated (Zweytick et al., 2000).

Chapter VI

TG-LIPASE EXPRESSION IN THE FAT BODY OF *M. SEXTA* DURING DEVELOPMENT

INTRODUCTION

Larvae of the tobacco hornworm, *Manduca sexta*, feeds constantly and stores carbohydrates and lipids as a major energy reserves in a very short time (Beenakkers et al., 1985; Arrese et al 1997). The larvae weight less than 1 mg when they hatch and 2 weeks later weight more than 10 g. The content of the lipids increases from few µg on hatching to ~80 mg at the end of the larval stage (Fernando-Warnakulasuriya et al., 1988). During the pupal period, these lipid reserves are used to support metamorphosis and at the adult stage to support the energy demands of flight and reproduction (Ziegler, 1991). Thus, during development the fat body tissue changes from lipid-storing tissue to a lipid-mobilizing tissue (Tsuchida and Wells, 1988).

The mobilization of these energy reserves depends upon the developmental stage and is under the hormonal regulation by the neuropeptide AKH. During the energy requiring activities, AKH mobilizes glycogen in the larval stage, whereas it promotes a massive lipolytic response in adult stage (Ziegler et al., 1990a). Also, AKH has been shown to mobilize lipids in *M. sexta* larva (Ziegler et al., 1995) and Locust (Mwangi and Goldsworthy, 1977); however the extent of lipids mobilized is small amount. Moreover, signal transduction of

AKH also causes the intracellular increase of the same second messengers, cAMP and calcium, in larvae and adult stage (Wang et al., 1990; Vroemen et al., 1995 and 1998; Arrese et al., 1999). These results indicate that identical AKH receptor and its second messengers exist in both stages of an insect. The down stream signal of AKH activates the respective enzymes in energy mobilization. In *M. sexta,* AKH has been shown to activate glycogen phosphorylase in the larval stage (Ziegler et al., 1990) and TG-lipase in the adult stage (Arrese et al, 1996). Moreover, Ziegler (1991) showed that AKH also activates glycogen phosphorylase in adult insects. However, nothing is known about the existence of TG-lipase in the larval fat body. Since we are interested in understanding mechanism of TG-lipase activation in adult *M. sexta*, it would be logical to ask, if TG-lipase is present in the larval fat body? If it is present, how AKH prevents its activation, since larvae does not mobilize lipids. This information will help us to understand the mechanism by which AKH regulates the fat body TG-lipase activity in the adult insect. Availability of antibody against TG-lipase and knowledge of TG-lipase activity assay prompted us to investigate the expression pattern of TG-lipase during the developmental stages of *M. sexta*.

EXPERIMENTAL PROCEDURES

Homogenate

All steps were carried out on ice or at 4 ºC. Fat body tissue from 5 insects was pooled and homogenized with a Potter-Elvehjem glass homogenizer fitted with Teflon pestle, using 6 ml of homogenization buffer (50 mM Tris, pH 7.4, 0.25 M sucrose, 2 mM EDTA, 0.2 mM benzamidine, 10mg/l leupeptin, 1 mg/l aprotonin, 2 mM Dithiothreitol). The homogenate was overlaid with 2 ml of buffer without sucrose, and subjected to ultracentrifugation at 65,000 x g for 1 hr. The infranatant (cytosolic fraction) was collected and analyzed for TG-lipase activity and protein levels or was passed through Q-sepharose column equilibrated with buffer (10 mM Na_2HPO_4, pH 7.4, 1 mM EDTA, 0.1% 2-mercaptoethanol (v/v), 0.1 mM benzamidine, 0.02 % Triton X-100, 10 mg/l leupeptin, 1 mg/l aprotonin). After extensive wash with equilibration buffer, proteins were eluted with a NaCl gradient (20-150 mM) in the same buffer. TG-lipase was eluted in one fraction with 300 mM NaCl in the same buffer.

Assay for TG-lipase activity

Lipase activity was assayed using micellar [^{3}H] triolein substrate as described previously (Arrese and Wells, 1994). The final assay volume of 0.1 ml contained 50 mM Tris, pH 7.9, 500 mM NaCl, 0.02 % defatted bovine serum albumin, 1 mM EDTA, 2 mM dithiothreitol, 0.22 mM [^{3}H]triolein (4 µCi/µmol), and 1 mM Triton X-100. The reaction was initiated by adding cytosolic fraction. The mixture was gently vortexed for 20 s and incubated at 37 ºC with constant shaking. After 30

min, the reaction was terminated by the addition of 500 µl of an extraction mixture consisting of chloroform/methanol/benzene (2:2.4:1), containing 0.1 µmol of unlabeled oleic acid as carrier. Then, 40 µl of 1 N NaOH were added to the mixture. The mixture was vortexed for 1 min and centrifuged at 2000g for 2 min. Aliquots of 150 µl from the upper aqueous phase were transferred to scintillation vials for counting. Enzyme activity was expressed as nmol of FFA formed /min/mg protein.

Western-blot

Polyclonal antibodies against purified TG-lipase were raised in chicken at Cocalico Biologicals (Reamstown, PA). For western blotting, proteins were separated by SDS-PAGE (8%) according to laemmli (1970), and transferred to nitrocellulose membranes. Immunodetection was performed using anti-TG-lipase antibody. After incubation of membrane with horseradish peroxidase-conjugated rabbit anti-chicken secondary antibody, peroxidase activity was detected using ECL chemiluminescence reagents (Amersham Biosciences).

RESULTS and DISCUSSION

Expression of fat body TG-lipase

The presence of TG-lipase activity during the development of *M. sexta* fat body was studies in tissue homogenate. Our results identified that TG-lipase activity is present in the larvae and adult insects (**Figure 28**). In the larval stage, TG-lipase activity increases as insect grows and the maximum activity was observed during early 5^{th} instar. The activity then decreases in late 5^{th} and wanderer stage, and finally no activity was detected during entire pupal stage. Since adult insect rely on lipids as a major energy source, we further plan to examine the expression of TG-lipase after eclosion. As shown if **figure 28**, we identified that minimal TG-lipase activity was present in 1 hr adult insect after eclosion and there was no significant increase in lipase activity upto 6 hr of adult life. The lipase activity then increases and reaches its peak at 48 hrs after eclosion. The TG-lipase activity in 48 hrs adult insect fat body was ~4 fold higher than that observed in 5^{th} instar larvae insects. TG-lipase activity in adult insects is in good aggrement with a previous study carried out in adult *M. sexta* (Ziegler, 1984). Using hemolymph lipid levels as an indicator of AKH effect, Ziegler (1984) showed that the adiopokinetic response of adult *M.sexta* develops several hours after the imaginal molt and no or a very week response is found during the first 8 hrs of the adult life. After 16 hrs of adult eclosion the insect is sensitive to AKH, but the response is even greater on the second day of adult life.

We also examined the TG-lipase protein levels during development using antibodies raised against purified TG-lipase from adult *M. sexta*. As shown in **figure 29**, the TG-lipase protein levels of larval samples coincide very well with the TG-lipase activity. These results indicate that the identical TG-lipase exist in larvae and adult insects. In adult insects, since TG-lipase is localized in the cytosol and has an electrophoretic mobility that is very close to that of the storage proteins (85% of cytosolic proteins), it was necessary to remove them. This was done by passing cytosol of each group of insects through an anionic exchanger resin (Q-sepharose) as indicated in Methods. **Figure 29** shows the TG-lipase protein levels of the fraction eluted from Q-Sepharose with 300 mM NaCl corresponding to the cytosol fraction obtained from adult insects after different time of eclosion. The TG-lipase protein levels coincide with the TG-lipase activity as observed in **figure 29**.

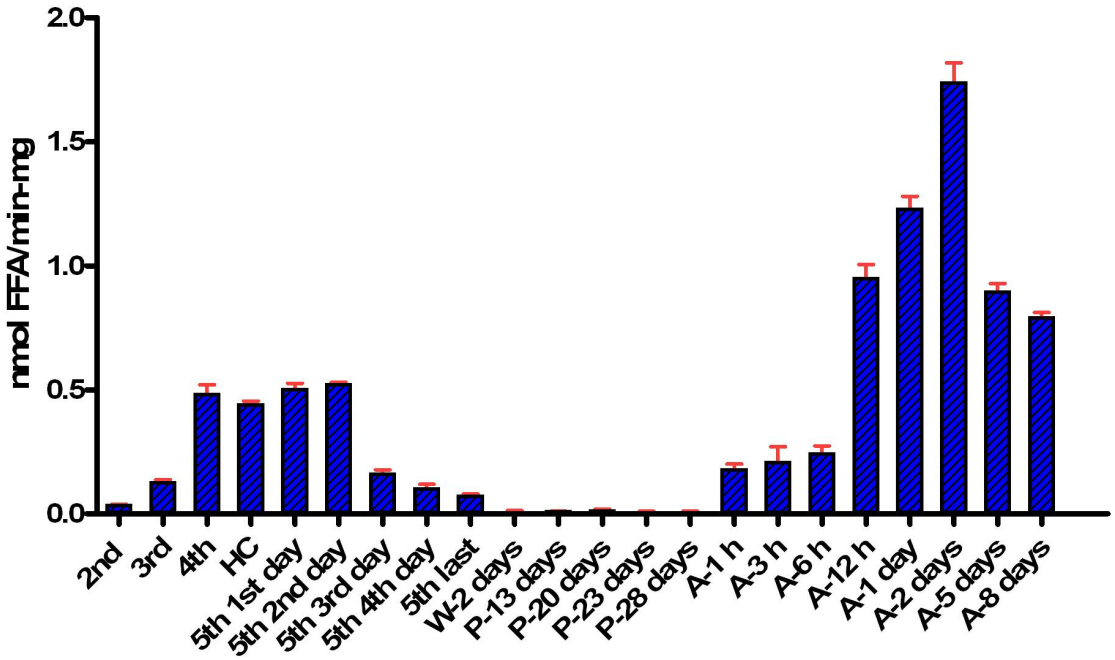


Figure 28: Changes in TG-lipase activity in the fat body of *M. sexta* during development: The cytosolic fraction after 65,000 x g centrifugation of larval, pupa and adult *M. sexta* fat body homogenates were examined for TG-lipase activity (200 µg) against an emulsion of [^{3}H-triolein] and Triton X-100 as indicated in Materials and Methods. Abbrevations: **2nd**, 2nd instar larvae; **3rd**, 3rd instar larvae; **4th**, 4th instar larvae; **HC**, head cup; **5th 1st day**, 5th instar 1 days old larvae; **W,** wanderer; **P,** pupa; **A**, adult; **A-1 h**, I h adult. Data represents means ± SE (n = 4).

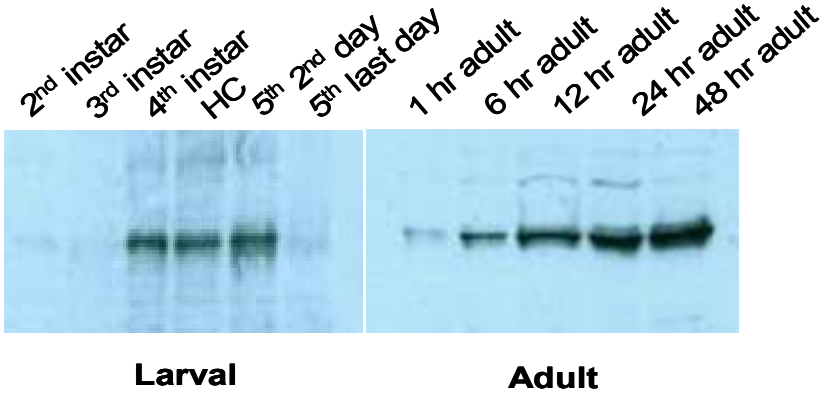


Figure 29: Changes in TG-lipase protein levels in the fat body of *M. sexta* during development: 40 µg protein from larval fat body homogenates and 20 µg protein from 300 mM NaCl elution after Q-Sepharose chromatography of adult fat body homogenates were separated on SDS-PAGE, transferred to nitrocellulose, and analyzed by Western blotting using anti-lipase antibody as indicated in Materials and Methods. Abbrevations: as shown in figure 28

The larva of tobacco hornworm, *M. sexta*, mobilizes mainly glycogen during energy demanding processes like starvation and molting. This is under the regulation of AKH. Moreover, AKH mobilizes very minimal amount of lipids in *M. sexta* larvae (Ziegler, et al., 1995). Since larval fat body contains 4 fold less TG-lipase compared to adult insects, but the effect of AKH on lipid mobilization in larval insects is very minimal compared to adult insects (from 8 mg/ml to 40 mg/ml, Ziegler, 1984), it seems very likely that AKH does not activate TG-lipase in larvae fat body.

We do not know the reason for the existence of TG-lipase in larval fat body, but molting larvae stop feeding for long times. This might influence the mobilization of lipids, the process which is independent of AKH. Also, the insect weight doubles during 5^{th} instar period, which might require large amount of lipids for cell membrane synthesis and energy for growth.

CHAPTER VII

TRANSACYLASE ACTIVITY OF TG-LIPASE

INTRODUCTION

Unlike vertebrates, where stored TG are mobilized as FFA, in insects, most, if not all mobilize stored TG as *sn*-1, 2-diacylglycerols (DG) (Beenakkers et al., 1985; Lum and Chino, 1990; Arrese and Wells, 1997). In the first step of lipid mobilization, TG must be hydrolyzed by the action of a lipase. This enzyme has been purified from fat body of *M. sexta* (Arrese and Wells, 1994). The *in vitro* hydrolysis of triolein catalyzed by the purified fat body TG-lipase produces *sn*-2-MG (Arrese and Wells, 1994). However, using an *in vivo* radiolabeled TG experiment it was shown that the content of MG in the fat body of *M. sexta* remains unchanged after stimulation of lipolysis by AKH (Arrese and Wells, 1997). Moreover, the reacylation of 2-MG seemed unlikely because monoacylglycerol-acyltransferase (MGAT) activity of the fat body is not affected by the stimulation of lipolysis. Thus it was concluded that the direct stereospecific hydrolysis of TG into *sn*-1, 2-DG is the pathway for DG synthesis in the fat body of *M. sexta* (Arrese and Wells, 1997). This was based on the observations that the only significant change among the fat body lipid components induced by AKH was an accumulation *sn*-1, 2-DG pool, whereas the free fatty acid (FFA), MG and

phosphatidic acid (PA) pool sizes remained unchanged. The de novo synthesis of DG from *sn*-glycerol-3 phosphate via phosphatidic acid pathway also seems highly unlikely because FFA and PA pools were unchanged (Arrese and Wells, 1997).

The discrepancy between the *in vivo* and *in vitro* experiments was attributed to an artifact of the experimental conditions, since *in vitro* enzyme activity was characterized using an artificial emulsion of TG that certainly does not have the structural and chemical properties of the native substrate of TG-lipase. We recently investigated the TG-lipase activity using *in vivo* radiolabeled native substrate of TG-lipase, the lipid droplets (Patel et al., 2005). Even under this condition, the purified fat body TG-lipase produced *sn*-2-MG as a major product instead of *sn*-1, 2-DG. Since the end product of *in vitro* TG-lipase activity using artificial and natural substrate is accumulation of 2-MG, and in *in vivo* condition stored TG is mobilized as *sn*-1, 2- DG, we hypothesize that TG is first hydrolyzed to *sn*-2-MG. Later, synthesized *sn*-2-MG are stereospecifcally transacylated (2-MG + 2-MG = sn-1, 2 DG + glycerol) to give rise to sn-1, 2 DG, the form in which stored TG are mobilized. Because lipases are known to catalyze acyl-CoA-independent transacylation reactions (Andrews et al., 1988; Jenkins et al., 2004) and given the proximity of TG-lipase during lipolysis, we consider the possibility that TG-lipase may be involved in the CoA-independent transacylation reaction between 2-MG pool to give rise to *sn*-1, 2 DG. To test this hypothesis, we examine the transacylase activity of TG-lipase using *sn*-2-MG as substrate.

EXPERIMENTAL PROCEDURE

Substrate Preparation

Radiolabeled *sn*-2-monooleoylglycerol was obtained from radiolabeled [tri-9,10-^{3}H(N)]oleoylglycerol using pancreatinc lipase (Brockman, 1981). Final reaction conditions were 20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$ and 6 mM Na-taurocholate. The reaction was initiated by adding pancreatic lipase (50 units, Sigma) in a final volume of 350 µl. The mixture was gently vortexed for 20 s and incubated at 39 º C with constant shaking. After 60 min, the reaction was terminated by the adding of 1500 µl of chloroform: methanol (2:1) and 20 µl of 6 N HCl. The mixture was vortexed for 1 min and centrifuged at 2000 x g for 2 min. The organic phase was collected, dried under a steam of nitrogen, and the lipids were separated by TLC on silica gel G impregnated with 1.2 % boric acid, using chloroform: acetone 96:4 (v/v) as the developing solvent (Arrese and Wells, 1997). Lipids were visualized using iodine vapors and the spot corresponding to *sn*-2-MG was scraped from the plate. After complete removal of iodine, 2-MG was eluted from silica with chloroform : methanol (4:1) or diethyl ether (Arrese and Wells, 1997). A small aliquot of the extract was used for determination of radioactivity by liquid scintillation counter. The remaining extract of 2-MG was used for transacylation reaction.

Assay for Acylglycerol Transacylase Activity

2-monoolein (Sigma) dissolved in chloroform were transferred to a large centrifuge tube, and solvent was removed under a steam of nitrogen. The reaction was initiated by adding purified TG-lipase (0.3 µg) in a final volume of 50 µl. Final reaction conditions were 200 mM Tris, pH 7.9, 500 mM NaCl, 0.02 % BSA, 2 mM DTT and 0.05 % Triton X-100. 1 nmol (6.4 µCi/µmol) of radiolabeled [^{3}H] 2-MG was added per reaction mixture. The mixture was gently vortexed for 20 s and incubated at 37 º C with constant shaking. After 45 min, the reaction was terminated by the adding of 300 µl of chloroform: methanol (2:1) and 5 µl of 6 N HCl. The mixture was vortexed for 1 min and centrifuged at 2000 x g for 2 min. The organic phase was collected, dried under a steam of nitrogen, and the lipids were separated by TLC on silica gel G plates using hexane: ethyl ether: formic acid (70:30:3) as the developing solvent (Arrese et al., 2001). The MG, DG, FFA and TG fractions were visualized by iodine vapors and scraped from the plates. After complete removal of iodine, the radioactivity associated with each fraction was determined by liquid scintillation counting. Blank reaction did not contain enzyme. Enzyme activity was expressed as nmol of product formed/ min-mg protein.

RESULTS

To determine if TG-lipase could catalyze the transacylase reaction, purified TG-lipase was incubated with 2-monoolein. The transfer of oleoyl moiety from 2-monoolein (donor) to 2-monoolein (acceptor) was examined, and the synthesis of diolein was determined. Our results identified that diolein was synthesized as a product in this incubation (Figure 30). This result suggests that fat body TG-lipase is capable of catalyzing transacylation reaction to form diolein. The other product formed was FFA, which represented 66% while DG represented 33%. We did not detect any radiolabeled TG, suggesting that DG is not an acyl acceptor.

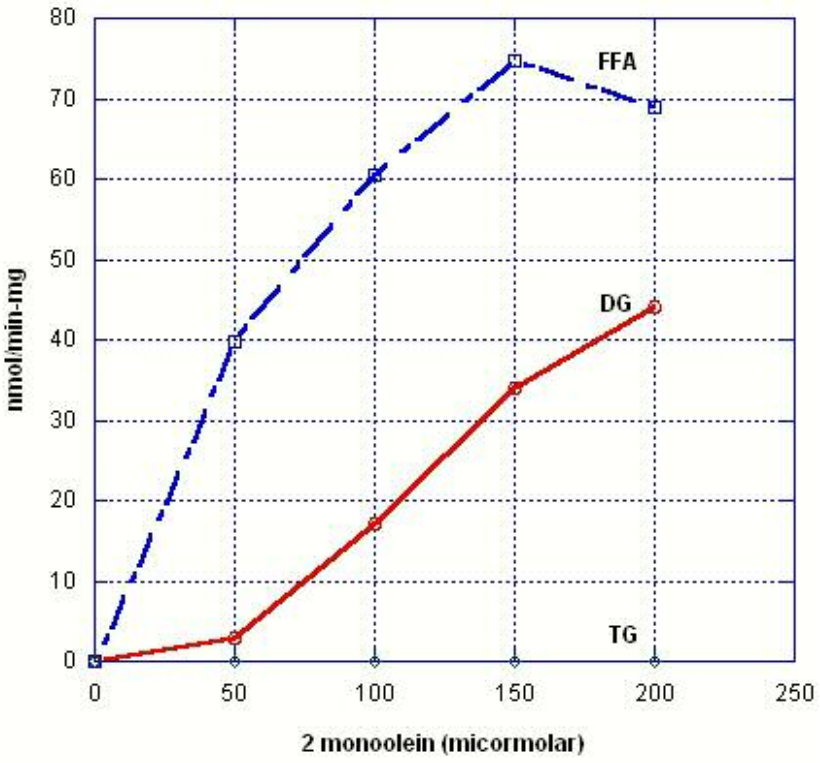


Figure 30: Highly purified TG-lipase catalyzes transacylase activity of 2-monoolein to form diolein. Assay was performed by incubating purified TG-lipase with varying concentration of [2-MG] as described under "Experimental Procedures. Radiolabeled products and remaining substrate were extracted into extraction mixture, resolved by TLC, and quantified by scintillation spectrometry".

DISCUSSION

Because lipases are known to catalyze transacylation reactions (Andrews et al., 1988; Jenkins et al., 2004), and because it has been shown that *in vitro* hydrolysis of triolein catalyzed by the purified fat body TG-lipase produces *sn*-2-MG (Arrese and Wells, 1994) instead of *sn*-1,2-DG the form in which stored TG are mobilized in insects (Arrese and Wells, 1997), we considered the possibility that fat body TG-lipase may be involved in the transacylation reaction for the re-synthesis *sn*-1,2 DG *in vivo*. The assay of purified TG-lipase with 2-mono-olein incubation resulted in DG formation, suggesting that TG-lipase has transacylase activity. We also observed FFA as another product of reaction mixture. The FFA formed was two fold higher compare to DG. The source of FFA could be the result of hydrolysis of 2-MG or the hydrolysis of DG formed during the reaction. Since *sn*-2-MG is a very poor substrate of *M.sexta* fat body TG-lipase (Arrese and Wells, 1994) and the rate at which FFA are formed in our experiment, it is very unlikely that 2-MG was hydrolyzed by TG-lipase. It has been shown that *M. sexta* fat body TG-lipase hydrolyzes DG at much higher rate than TG. The V_{max} for DG is two times that of TG (Arrese and Wells, 1994). These results support the conclusion that the source of FFA formed in transacylase reaction is the hydrolysis of DG. We did not detect any TG formation, suggesting that DG is not an acyl acceptor in transacylation reaction we examined.

Recently, four new TG-lipases from vertebrate adipocytes have been disclosed (Soni et al., 2004; Zimmermann et al., 2004; Jenkins et al., 2004).

Three of them, adiponutrin, TTS-2.2 (ATGL), and GS2, have high TG-lipase and acyl-transacylase activity (Jenkins et al., 2004). In vertebrates, adipose triglyceride lipase (ATGL) has been implicated as the rate limiting enzyme in adipocytes lipolysis (Zimmermann et al., 2004). It has been envisaged that the presence of anabolic (transacylase) *versus* catabolic (lipase) activities provides a potential mechanism to rapidly switch cellular metabolic balance from energy storage to mobilization. The transacylase activity of *M. sexta* TG-lipase examined in this study is also ATP and Acyl-CoA independent. These dual activities of *M. sexta* TG-lipase could play an important role in regulating DG being mobilized from the fat body cell.

Three different mechanisms were proposed for the stereospecific synthesis and secretion of *sn*-1, 2 DG: a) hydrolysis of TG into *sn*-2-MG followed by stereospecific acylation of *sn*-2-MG catalyzed by MGAT. MGAT activity is found in the fat body of L. *migratoria* (Tietz et al., 1975), *Periplaneta Americana* (Hoffman and Dower, 1979) and *M. sexta* (Arrese et al., 1996), b) de novo synthesis of DG from *sn*-glycerol-3 phosphate via phosphatidic acid (PA) using fatty acid produced by TG hydrolysis (Tietz et al., 1975; Arrese and Wells, 1994) c) the stereospecific hydrolysis of TG into *sn*-1, 2-DG (Spencer and Candy, 1976; Lum and Chino, 1990; Arrese and Wells, 1994). Although MGAT activity is observed in insect fat body, no effect of AKH on MGAT activity was observed on stimulation of lipolysis in *M.*sexta (Arrese et al., 1996). Moreover, the content of MG, FFA and PA in the fat body of *M. sexta* remains unchanged after stimulation

of lipolysis by AKH (Arrese and Wells, 1997). Additionally, the end product of *in vitro* TG-lipase activity using artificial and natural substrate is accumulation of 2-MG (Arrese and Wells, 1994; Patel et al, 2005). Thus, in present study, we propose the fourth possibility for the synthesis of *sn*- 1,2 DG. Because no significant change in *sn*-2-MG pool was observed on stimulation of lipolysis by AKH (Arrese and Wells, 1997), the rate of TG hydrolysis should be similar to rate of transacylase activity for this pathway to exist in cell. The maximum concentration of substrate used for transacylase reaction in this study was 200 µM, which did not prove to be optimal. Further work with higher concentration of 2-MG is required to obtain saturation kinetics. Also, the hydrolysis of DG formed by transacylation reaction should be prevented to have a better comparison of both the activities. Altogether our results suggest that TG-lipase has transacylase activity and might play a role in sn-1, 2 DG synthesis.

CHAPTER VIII

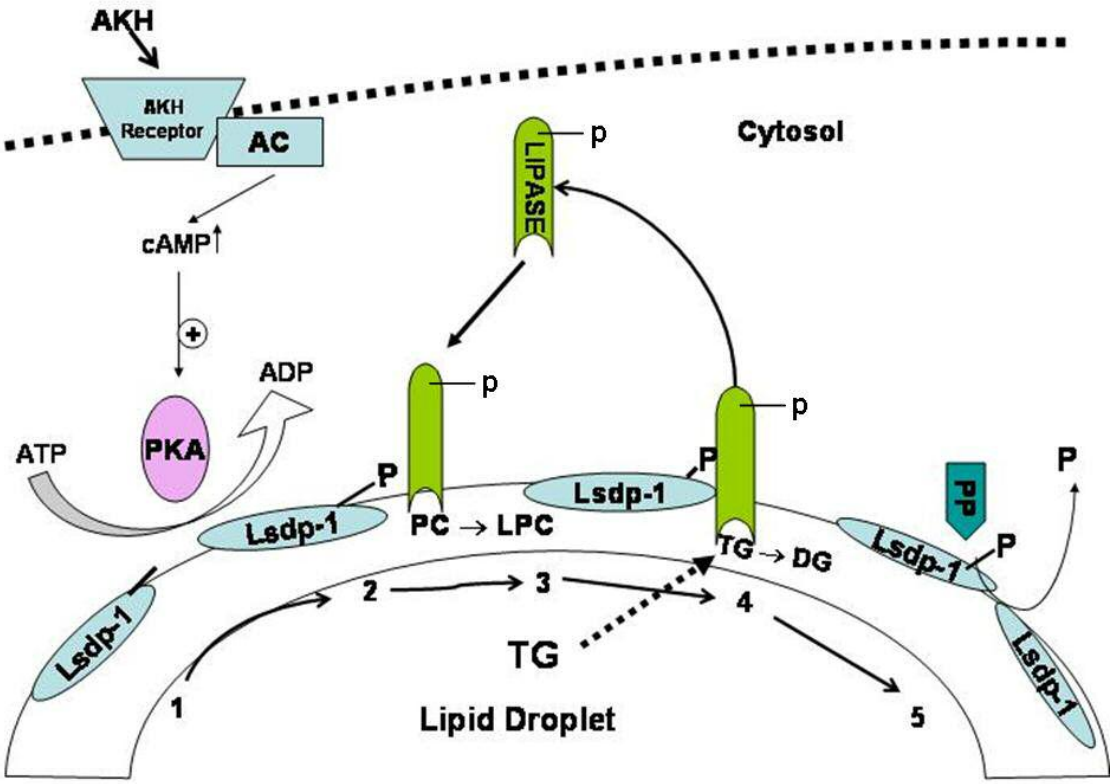
SUMMARY

In the present study we show that lipolysis in fat body adipocytes is mainly controlled by activation of the substrate. This activation is achieved by PKA mediated phosphorylation of the lipid droplet-associated proteins. The changes in the phosphorylation of Lsdp1 correlated with the increase in TG-lipase activity suggesting that this protein is a major player in the activation of lipolysis in insects. In contrast to previous understanding, that AKH might regulate lipolysis by regulating TG-lipase activity by phosphorylation/dephosphorylation mechanism, here we show that, AKH does not change the state of phosphorylation of TG-lipase on stimulation of lipolysis. We further show that TG-lipase has phospholipase activity, which could play an important role in the hydrolysis of mono layer of phospholipids present on the surface of the lipid droplets.

Several questions remain to be addressed to fully understand the mechanism of regulation of lipolysis in insects. Understanding the role of Lsdp1 in the molecular mechanism of activation of the lipid droplets and lipolysis constitutes a challenge of future studies. AKH stimulates the phosphorylation of more than one protein on the surface of the lipid droplets. The role/s of those proteins in the mechanism of lipolysis needs to be elucidated. Once DGs are formed, they are likely to be transferred to a carrier. TG-lipase could interact with

such a carrier which would bind DG preventing its hydrolysis and transporting to the membranes for its export to the hemolymph. TG-lipase could be interacting with other proteins through the WWE domain which is involved in specific protein-protein interactions. The role of this domain in the function of the lipase is unknown. The enzyme could associate with other partner/s perhaps with some implication in the regulation of its activity.

Figure 31. Current model for the mechanism of AKH-induced lipolysis



The mechanisms of basal and stimulated lipolysis are complex processes whose details are not fully understood in any system. The complexity of the

process derives from the low solubility of the substrate and its products in aqueous medium which impose specialized mechanisms presumably involving multiple steps and proteins. The current information on the mechanism of AKH-induced lipolysis in *M.sexta* anticipates the course of events that is depicted in **Figure 31**.

Binding of the hormone AKH to its receptor triggers activation of the adenylate cyclase (AC) and concomitant increase in the cAMP concentration followed by PKA activation (Arrese et al., 1999). PKA has multiple targets; however, phosphorylation of Lsdp-1 is the main target related to activation of lipolysis (Step1, Patel et al., 2005). Phosphorylation of Lsdp-1 enhances binding of the lipase to the surface of the lipid droplet and/or its catalytic activity (Step 2). Lipid droplet bound lipase catalyzes the hydrolysis of phospholipid (Step 3) allowing the access of TG to the lipid surface (step 4) and its subsequent hydrolysis also catalyzed by the lipase. The lipolytic process ends by release of the lipase form the lipid surface. Note that, unlike HSL (Brasaemle et al., 2000; Holm, 2003), the insect TG-lipase does not bind tightly to the lipid droplets, even under conditions of high lipolysis (Patel et al., 2005). Additional binding of the lipase to the lipid droplet would be prevented by de-phosphorylation of Lsdp-1 mediated by a protein phosphatase (PP).

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Pages in Study: 140 Candidate for the Degree of Doctor of Philosophy

Major Field: Biochemistry

Scope and Method of Study:

Insects are the largest group of organism on the planet. Because of economical and ecological reasons, it is important to understand insects. The main aim of this study was to advance the knowledge of lipid metabolism using *Manduca sexta* as a model organism. *In vivo* and *in vitro* experiments were used to investigate the intracellular signaling mechanisms involved in the activation of triglyceride (TG) lipase by the lipid mobilizing hormone, proteins involved in the TG hydrolysis, and characterization of TG-lipase.

Findings and Conclusion:

Our results identified that adipokinetic hormone (AKH) stimulates lipolysis by highly phosphorylating “Lipid Storage Droplet Protein 1” (Lsdp1) of the lipid droplets and increase in TG-lipase activity of cytosol. The phosphorylation of Lsdp1 is important for lipolysis and is mediated by cAMP dependent protein kinase (PKA). The changes in the phosphorylation of Lsdp1 correlated with the increase in TG-lipase activity suggesting that this protein is a major player in the activation of lipolysis. Although AKH increases TG-lipase activity of cytosol isolated from hormone stimulated insects compare to control insects, it does not change the state of phosphorylation of TG-lipase, indicating that phosphorylation of the TG-lipase plays no role in the activation of lipolysis. Overall, the changes in the lipid droplets are responsible for ~70% of the lipolytic response to AKH. The remaining 30% appears to be due to AKH-dependent changes in cytosol. We further identified that TG-lipase has phospholipase A_1 activity and is able to hydrolyze phospholipids present on the surface of the lipid droplets. Altogether, it is concluded that lipolysis in fat body adipocyte is mainly controlled by activation of the substrate.

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