The signal transduction of the adipokinetic hormone and regulation of energy metabolism in the cricket, *Gryllus bimaculatus* de Geer (Ensifera: Gryllidae)

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To, my family and teachers

Abbreviations

ACCase	acetyl-CoA carboxylase
ACL	ATP:citrate lyase
AKH	adipokinetic hormone
apoLp-III	apolipophorin III
CA	corpora allata
Ca_e^{2+}	extracellular calcium
Ca_i^{2+}	intracellular calcium
CC	corpora cardiaca
DAG	diacylglycerol
FA	fatty acid
FAS	fatty-acid synthase
FBIM	fat body incubation medium
HB	homogenization buffer
HDLp	high-density lipophorin
HSL	hormone-sensitive lipase
IP_3	inositol 1,4,5-trisphosphate
LDLp	low-density lipophorin
LL	last larval instar
MAG	monoacylglycerol
MM199	modified Medium 199
PKA	protein kinase A
PKC	protein kinase C
PL	penultimate larval instar
PLC	phospholipase C
TAG	triacylglycerol
TGL	triacylglycerol lipase
TLC	thin layer chromatograph

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1. Introduction

Insects, the most diverse group of animals on the earth, play a very important part in ecosystems. Their roles include nutrient recycling, plant pollination, maintenance of plant community composition and structure, and supporting insectivorous animals. They provide us with many useful materials such as honey, silk, dye, varnish, chitin and so on. Of course, some insects are harmful to humans and domestic animals and adversely affect agriculture, making the study of insects even more important.

The importance of insects as biochemical models is supported by the fact that many discoveries in insects are applicable to vertebrate systems as well (Law and Wells, 1989). The basic physiological processes of digestion, muscle contraction, and nervous transmission, as well as important metabolic and developmental pathways, are almost identical in insects and vertebrates. Insects have short life-cycles, they are cheap and easy to maintain in the laboratory, and they are available in a huge variety and number. Thus, they are very useful models for understanding biological processes in general (Klowden, 2003). Research on insects has immensely contributed towards our understanding of neuroendocrinology. More studies on structure, metabolism, release, receptor binding, mode of action and analogues of neuropeptides in insects would be useful in developing target specific and eco-friendly insecticides (Couillaud and Peypelut, 1995; Hoffmann et al., 2001; Gäde and Goldsworthy, 2003).

Insects possess an open circulatory system, where the hemolymph is contained in the general body-cavity, bathing various tissues. Between 15 and 75% of the volume of an insect is made up by hemolymph, the amount and composition of which varies with species and its physiological condition (Richards and Davies, 1994). They have developed special systems for efficient and fast use of energy stores in processes like reproduction, embryogenesis, metamorphosis and flight.

Flying ability is one of the primary reasons that insects have been successful in nature. Flight assists insects in escaping from danger, finding food, locating mates and dispersal. It involves the highest metabolic rate in nature, which increases 50 to 100-fold compared to that at rest (Beenakkers et al., 1984; Beenakkers et al., 1985a). The dramatic increase in the fuel demand is accomplished by fat body, which is the main storage organ of highly energy-rich molecules.

1.1. Fat body

The insect fat body is made up of aggregates of cells, forming lobes or sheets of highly tracheated tissue suspended in the hemolymph, thus ensuring ready access to nutrients, proteins, and hormones (Beenakkers et al., 1985b). The fat body is mainly composed of adipocytes, which function in the uptake and storage of nutrients. In addition, other fat body cells are specialized as mycetocytes (which host bacterial symbionts) and urocytes (which store and excrete uric acid). The fat body is the major center for intermediary metabolism, which involves the homeostatic maintenance of hemolymph proteins, lipids, and carbohydrates. The fat body also performs developmentally specific metabolic activities that produce, store, or release components central to the prevailing nutritional requirements or metamorphic events of the insect. Storage protein is synthesized in the fat body and secreted into the hemolymph, making up 75-80% of the total hemolymph protein (Wyatt and Pan, 1978). Later, the protein is reabsorbed into the fat body and stored in the form of dense protein granules, which can be used as a source of amino acids and energy. During vitellogenesis, the fat body plays a vital role in vitellogenin synthesis (Hoffmann, 1995). Additionally, lipids are transported from the fat body to the ovaries for egg development. Thus, the fat body is functionally analogous to both vertebrate liver and adipose tissue (Beenakkers et al., 1985b).

1.2. Flight fuels

On initiation of flight, the fat body releases carbohydrates, lipids, the amino acid proline, or mixtures thereof into the hemolymph, which are oxidized by flight muscles to fuel flight (Gäde and Auerswald, 2003).

1.2.1. Carbohydrates

Carbohydrates are stored in the fat body mainly in the form of glycogen, which can be rapidly hydrolyzed to release trehalose into the hemolymph (Wyatt, 1967; Candy, 1985; Candy et al., 1997; Thompson, 2003). Trehalose is transformed into glucose and used by the flight muscles when energy is required. Trehalose can be synthesized in the fat body from monosaccharides of dietary origin or by gluconeogenesis from a variety of precursors. It is the major sugar in insect hemolymph and the most important for flight. It is a non-reducing, less reactive sugar and therefore has an advantage over glucose and other reducing sugars. It occurs in the hemolymph in concentrations between 5 and 50

mM, which differ considerably between species. Regulation of trehalose synthesis occurs at two levels: hormonal regulation of glycogen conversion to trehalose and feedback inhibition by trehalose. Insects belonging to the orders Hymenoptera, Diptera and Blattodea are examples of insects using carbohydrates as fuels for flight.

1.2.2. Lipids

Lipids are highly reduced, and therefore hold more energy per gram than glycogen. In addition, lipids can be stored in compact form, as they have very little water associated to them compared to glycogen. Therefore, migratory insects which fly for long distances, generally use lipids to fuel flight (Cheeseman et al., 1976; Ziegler and Schulz, 1986). Hemolymph lipid content and composition vary with the physiological state of the animal. Diacylglycerol (DAG) is the major lipid component of hemolymph in most insect species (Beenakkers et al., 1985b). Hormonal activation leads to the release of lipids from the fat body, which are oxidized by flight muscle cells (Candy et al., 1997). In locusts, the initial stage of a flight is mainly based on fuels stored in the muscle cell, phosphoarginine and muscle glycogen, which are rapidly supplemented by hemolymph trehalose (Wegener, 1996). During prolonged flight, fuel utilization is gradually shifted from carbohydrates to the oxidation of lipids that are mobilized from locust fat body. Storage of energy in the form of lipids is also advantageous for insects that do not feed as adults. In insects, the lipid content can vary from 1-50% of the wet weight, and is influenced by many factors including stage of development, nutritional state, sex, environmental temperature, diapause and migratory flight. In most insects studied, triacylglycerol (TAG) comprises more than 90% of total fat body lipid. Several studies have indicated that fatty acid (FA) profile of the fat body is influenced by the FA composition of the diet (Beenakkers et al., 1985b).

1.2.3. Ketone bodies

The hemolymph of the locust, *Schistocerca gregaria*, has been shown to contain acetoacetate and smaller amounts of 3-hydroxybutyrate (Bailey et al., 1971). Ketone bodies appear to be good energy substrates for brain in insects as they are in mammals (Candy et al., 1997). Acetoacetate might be important during flight in locusts, as its concentration in the hemolymph increases during flight or upon injection with corpora cardiaca (CC) extracts. Ketone bodies are also present in *Periplaneta americana* hemolymph. However, not much is known about the use of ketone bodies in other species and more work is needed to confirm role of ketone bodies during flight.

1.2.4. Amino acids

During insect flight a number of amino acids can be oxidized to provide energy; in a number of insects, especially beetles, proline is the most important. Proline oxidation by the tsetse fly, *Glossina morsitans* flight muscle was first discovered by Bursell (1963, 1981). Later, it was found that in many beetles proline exclusively or in combination with carbohydrates is utilized as a fuel for flight activity (Weeda et al., 1979; Gäde and Auerswald, 2002). Proline is partially oxidized in the muscle cells, and the alanine which is formed in the process is transported to the fat body where it is reconverted to proline. The two carbon atoms required for the reconversion are derived by β–oxidation of FAs to acetyl-CoA. Regulation of proline synthesis is under hormonal control (Candy et al., 1997). Use of proline as a flight fuel has many advantages. It can be stored at high concentrations in flight muscles and hemolymph. Conversion of proline into alanine and vice versa does not release ammonia and no specific carrier molecules are necessary for their transport.

1.3. Adipokinetic peptides

The mobilization of energy-rich molecules from fat body reserves is brought about by the action of multifunctional peptide hormones belonging to the adipokinetic/hypertrehalosemic hormone family (for simplicity referred as AKH peptides) (Gäde et al., 1997; Gäde and Auerswald, 2003; Van der Horst, 2003). The structure of AKH was determined for the first time from the locusts *Schistocerca gregaria* and *Locusta migratoria* (Stone et al., 1976). To date, the structures of around 40 different peptides of this family are known from most of the major insect orders with seemingly overlapping functions. They are structurally very similar to the red pigment-concentrating hormone (RPCH) of crustaceans (Fernlund and Josefsson, 1972). In general, they are 8 to 10 amino acids long, N-terminally blocked by a pyroglutamate, and C-terminally amidated, thus protected from exopeptidases. They contain Trp and Gly at positions 8 and 9, respectively, and at least one additional aromatic amino acid, Phe or Tyr, at position 4. One of the two AKHs from the butterfly *Vanessa cardui* is unusual, having a length of 11 amino acids and a non-amidated COOH-terminus (Köllisch et al., 2000). Information on the physiological actions and roles that AKHs play, is available mainly for locusts, moths, beetles and cockroaches. Not much is known for most other species from which they have been isolated.

The pleiotropic nature of the AKH peptides is evident by their actions on many physiological processes. In addition to their role in energy mobilization, they inhibit protein synthesis (Carlisle and Loughton, 1979, 1986; Asher et al., 1984; Cusinato et al., 1991), inhibit mRNA synthesis (Kodrík and Goldsworthy, 1995), modulate neuro- or myoactivity (Scarborough et al., 1984; Witten et al., 1984; Milde et al., 1995; Socha et al., 1999; Kodrík et al., 2000), inhibit glycolysis (Becker and Wegener, 1998), and inhibit lipid synthesis (Gokuldas et al., 1988; Ziegler, 1997; Lorenz, 2001). The inhibitory action on lipid synthesis is analogous to that of glucagon in vertebrates. Interestingly, in locusts AKH not only inhibits general protein synthesis but preferentially inhibits vitellogenin synthesis (Moshitzky and Applebaum, 1990). AKH also inhibits protein synthesis in the aorta and midgut, which are not involved in lipid mobilization (Carlisle and Loughton, 1986), suggesting that receptors for AKHs are present in many tissues. Recent work by Goldsworthy and coworkers has pointed towards an additional effect in immune response (Goldsworthy et al., 2002a; Goldsworthy et al., 2003). These actions can have significant impact on insects but their exact roles need to be determined.

1.3.1. Synthesis and secretion of AKH

Stimulation of glycogen breakdown by injection of the extracts of the CC into the American cockroach was first demonstrated by Steele (Steele, 1961). This was the first evidence that intermediary metabolism in insects was under hormonal control. The CC, paired glands located caudally to the brain, are major neurohemal organs. They are physiologically equivalent to the pituitary gland of mammals. The glandular lobe of the CC synthesizes AKH which is stored in the storage lobe (Gäde et al., 1997).

L. migratoria has three AKHs encoded by distinct mRNAs. The signals for the mRNA of all three AKH preprohormones are co-localized in the neurosecretory cells of the glandular lobes of the CC (Bogerd et al., 1995; Van der Horst, 2003). Immunocytochemical studies have suggested that individual neurosecretory cells contain all three AKH peptides and that they are co-localized in the same secretory granules (Diederen et al., 1987; Harthoorn et al., 1999), implying their simultaneous release upon stimulation. The AKH-producing cells (AKH cells) continuously synthesize AKH and the synthesis is not affected by its release during flight (Harthoorn et al., 2001). Flight activity is the only known natural stimulus for the release of AKH. A very small fraction of the AKH stored is released during flight and the granules containing newly synthesized AKH only appear to be available for the release

(Sharp-Baker et al., 1995, 1996; Harthoorn et al., 2002). Locustatachykinins and crustacean cardioactive peptide (CCAP) stimulate AKH release from the CC *in vitro*, while trehalose has an inhibitory effect (Vullings et al., 1999; Van der Horst et al., 1999). There are indications that locustamyoinhibiting peptide and FMRFamide-related peptides play a role in the inhibition of AKH release from the CC (Vullings et al., 1998; Harthoorn et al., 2001).

Secretion of AKH by the CC is believed to be controlled by conventional neurons from the brain, since severing the nervous connection between brain and CC prevents the flight-induced elevation of hemolymph lipid titers (Goldsworthy et al., 1972), while electrical stimulation of these nerves in isolated CC causes the release of AKH (Orchard and Loughton, 1981). AKHs are transported in the hemolymph without any carrier (Oudejans et al., 1996). To terminate the signal, the peptides of AKH/HrTH family are cleaved by endopeptidases into fragments, which are susceptible to further degradation by exopeptidases (Isaac, 1988; Rayne and O'Shea, 1992; Oudejans et al., 1996).

1.3.2. Signal transduction of AKH

Recently AKH receptors have been cloned from the fruitfly *Drosophila melanogaster* and the silkworm *Bombyx mori* (Park et al., 2002; Staubli et al., 2002). The receptors are G-protein-coupled and are structurally and evolutionarily related to the gonadotropin-releasing hormone receptors from vertebrates. More than one such AKH receptor type can be present in an insect (Staubli et al., 2002). AKH peptides are believed to act by binding to the receptor which changes conformation and interacts with a G-protein. This, subsequently, transduces the signal to an enzyme, which produces a second messenger in the cytoplasm. Signal transduction of AKH peptides has only been studied in a few insects and many details remain far from clear, nevertheless a general picture has been deduced (Van der Horst et al., 1999; Gäde and Auerswald, 2003).

In general, in hypertrehalosemia, peptides bind to the G-protein-coupled-receptor and activate phospholipase C (PLC) increasing inositol 1,4,5-trisphosphate (IP_3) levels. This induces the release of Ca^{2+} from intracellular Ca^{2+} stores which leads to the initiation of the capacitative Ca^{2+} entry into the cytosol. The increased Ca^{2+} concentration results into phosphorylation and activation of glycogen phosphorylase by phoyphorylase kinase. AKH further enhances the efflux of Ca^{2+} from the cytosol to reach the normal basal level. In *P.*

americana, production of DAG along with IP_3 has been proposed. DAG in conjunction with Ca^{2+} then activates protein kinase C (PKC), which, in turn, activates glycogen phosphorylase by phosphorylation (Sun and Steele, 2001; Sun and Steele, 2002).

In general, in hyperlipemia, binding of AKH leads to a conformational change in a G_s-protein which, in turn, activates an adenylate cyclase, resulting in an increase of intracellular cAMP levels. Cyclic AMP stimulates lipase activity, most likely via activation of a protein kinase A (PKA). The influx of extracellular Ca^{2+} is also essential for the adipokinetic effect. In moths, release of Ca^{2+} from IP_3-insensitive intracellular stores causes an increase in the hemolymph lipid titers (Arrese et al., 1999).

The mode of action of AKH during hyperprolinemia appears to be similar to that during hyperlipemia. It seems that AKH binds to the receptor to cause a conformational change of a G_s-protein which, in turn, activates an adenylate cyclase. The increase in cAMP levels might activate triacylglycerol lipase (TGL) and consequently TAG breakdown to release FAs. AKH seems to activate Ca^{2+} release from intracellular stores and also the capacitative Ca^{2+} entry into the cytosol. Free FAs produced can undergo β-oxidation, the resulting acetyl-CoA, together with alanine, are used for re-synthesis of proline.

1.4. Octopamine

Octopamine, a biogenic amine, closely related to the catecholamine noradrenaline, acts as a neurotransmitter, neuromodulator and/or neurohormone in many invertebrates. Octopamine can affect all organs of insects (Candy et al., 1997). In insect hemolymph, octopamine can reach concentrations of 2×10^{-7} M (Goosey and Candy, 1980). Hormonal effects have been observed in isolated fat bodies. High hemolymph concentrations of octopamine have been reported in the early stage of locust flight and as a consequence of stressful situations, such as handling, in cockroaches and crickets (Davenport and Evans, 1984; Woodring et al., 1989; Fields and Woodring, 1991). In both situations, the fat body mobilizes stores and releases fuels into the hemolymph. Octopamine increases the force and efficiency of flight muscle contractions and also stimulates the oxidation of carbohydrate and fat in flight muscle preparations (Candy, 1978; Whim and Evans, 1988; Orchard et al., 1993). In locusts, octopamine potentiates the release of AKH from the CC induced by cAMP agonists (Pannabecker and Orchard, 1986; Passier et al., 1995). In many

insects, hemolymph lipid concentration increases under starvation and not only during flight. The increase in the lipid concentration during starvation is independent of AKH, in contrast to that induced during flight (Beenakkers et al., 1985b) and may be due to octopamine action.

1.5. Enzymes of lipid metabolism

Lipid synthesis and mobilization are very important processes in insect physiology. They are well regulated and highly complex processes involving a battery of enzymes. Some of the key enzymes involved are acetyl-CoA carboxylase (ACCase), ATP:citrate lyase (ACL), fatty-acid synthase (FAS) and triacylglycerol lipase (TGL).

ACCase catalyzes the formation of malonyl-CoA from acetyl-CoA, the rate-limiting and first committed step in the synthesis of long-chain FAs (Wakil et al., 1983; Hardie, 1989). The enzyme is located in the cytosol (Storey and Bailey, 1978) and has two isoforms, ACCα and ACCβ (Munday, 2002). The isoforms are regulated by short-term mechanisms of allosteric activation by citrate and phosphorylation/dephosphorylation. AMP-activated protein kinase seems to be involved in the phosphorylation and inactivation of both isoforms (Munday et al., 1988). ACCase gene expression is under nutritional and hormonal control (Kim, 1997).

ACL is a key enzyme in the conversion of carbohydrate into lipid. It catalyzes the synthesis of cytoplasmic acetyl-CoA, necessary for lipogenesis (Srere, 1959). Activity of ACL decreases upon phosphorylation by the cAMP-dependent protein kinase (Houston and Nimmo, 1985).

FAS, a multifunctional enzyme, synthesizes FAs in the cytoplasm. It catalyzes seven enzymatic reactions to produce palmitate from acetyl-CoA and malonyl-CoA (Wakil et al., 1983; Wakil, 1989). The FAs produced are used for the synthesis of TAG. FAS activity is regulated by nutrients or hormones and not by allosteric effectors or covalent modification (Sul and Wang, 1998). In vertebrates, glucagon inhibits expression of FAS gene via the increase in intracellular cAMP (Sul et al., 1998).

In insects that utilize lipid as a fuel for flight activity, TGL plays a crucial role in the mobilization of lipid from fat body stores (Ryan and Van der Horst, 2000). TGL catalyzes the degradation of TAG leading to the release of DAG from the fat body into the hemolymph. In vertebrates, TAG is hydrolyzed by hormone-sensitive lipase (HSL) (Nachman et al., 1997). HSL is activated by phosphorylation via cAMP-dependent PKA. The activation might involve both conformational change in the HSL and translocation of HSL from the cytosol to the lipid droplet (Egan et al., 1992).

1.6. Lipid breakdown and mobilization

Upon AKH stimulation, TAG is hydrolyzed and *sn*-1,2-DAG is released from the fat body into the hemolymph (Lok and Van der Horst, 1980; Tietz and Weintraub, 1980; Arrese and Wells, 1997). The mechanism of *sn*-1,2-DAG formation from TAG is not yet clear, as there are conflicting reports. Three different pathways for the formation of *sn*-1,2-DAG have been proposed: 1. The monoacylglycerol (MAG) pathway - the hydrolysis of TAG into 2-MAG followed by acylation of 2-MAG (Chino and Gilbert, 1965; Tietz et al., 1975; Tietz and Weintraub, 1978) 2. The *sn*-3-glycerophosphate pathway - *de novo* synthesis of DAG from glycerol-3-phosphate via phosphatidic acid using the FAs released by TAG hydrolysis (Arrese and Wells, 1994) and 3. the stereospecific hydrolysis of TAG into *sn*-1,2-DAG (Spencer and Candy, 1976; Lum and Chino, 1990; Arrese and Wells, 1997).

Insects have developed a highly efficient lipid transport system which involves a reusable lipid shuttle system (Ryan and Van der Horst, 2000). Hemolymph generally contains a single major lipoprotein particle, lipophorin, as a lipid transport vehicle. In addition to DAG, the high-density lipophorin (HDLp) can transport a variety of lipophilic molecules in the hemolymph (Trowell et al., 1994; Gu et al., 1995; Sevala et al., 1997; Schal and Sevala, 1998; Tsuchida et al., 1998). Exchangeable apolipoprotein (apolipophorin III, apoLp-III) and DAG released from the fat body cells are loaded on HDLp, to form a low-density lipophorin (LDLp). The process is facilitated by a lipid transfer particle (Canavoso and Wells, 2001; Golodne et al., 2001). LDLp carries the lipids to flight muscles where DAG is hydrolyzed by a lipophorin lipase and the FAs released are taken up and oxidized to provide energy. The glycerol released in the process is transported back to the fat body, where it can be used in the formation of new DAG or converted into trehalose by gluconeogenesis (Candy et al., 1997). The delivery of lipid to tissues, for the most part,

occurs without internalization and destruction of lipophorin, in contrast to the vertebrates (Canavoso et al., 2001). Stripping of lipids from the carrier molecule leads to the dissociation of HDLp and apoLp-III which are transported back to the fat body for reuse.

1.7. What is known in the cricket, *Gryllus bimaculatus*?

The regulation of development and reproduction by juvenile hormones and ecdysteroids in the cricket, *G. bimaculatus* has been well studied (Lorenz et al., 1997, 1998, 1999). By conventional purification methods five FGLamides (A type allatostatins) with allatostatic activity have been identified from the cricket (Lorenz et al., 1995b, 1999). cDNA cloning of the allatostatin gene of the cricket revealed the presence of 14 allatostatins (Meyering-Vos et al., 2001). Five W^2W^9-allatostatins (B type allatostatins) have been purified by conventional purification methods (Lorenz et al., 1995a, 1999; Hoffmann and Lorenz, 1997; Hoffmann et al., 1998). On the other hand, little information is available on the physiological role of AKH in the regulation of development and reproduction in the cricket, as well as in other insects.

The *G. bimaculatus* AKH (Grybi-AKH) is structurally related to the Schgr-AKH II (Gäde and Rinehart, 1987). It is an octapeptide with a primary structure of pGlu-Val-Asn-Phe-Ser-Thr-Gly-Trp-NH_2. The acetate incorporation into lipid by fat body, is inhibited in an age-dependent manner by AKH (Lorenz, 2001). The injection of AKH into adult and larval instar crickets induces an increase in the carbohydrate and lipid titers of the hemolymph (Woodring et al., 2002). Repeated injections of AKH into adult crickets inhibited the formation of energy stores in the fat body and consequently egg production (Lorenz, 2003). The increase in the hemolymph lipid titer at the beginning of scotophase coincides with an increased locomotor activity of crickets. Similar effects are elicited during photophase by injection or topical application of AKH (Lorenz et al., 2004). Thus, AKH might play an important role in the behavior, development and reproduction of crickets.

2. Materials and methods

2.1. Insects

2.1.1. *Gryllus bimaculatus*

A colony of Mediterranean field crickets, *Gryllus bimaculatus* de Geer (Ensifera: Gryllidae) was maintained in our laboratory. This consisted of crickets provided by the company b.t.b.e. Insektenzucht (Schnürpflingen, Germany) crossed with crickets collected in wild in Japan (a generous gift of Dr. S. Tanaka). The crickets were reared under long-day conditions (16 h light: 8 h dark) at a constant temperature of $27 \pm 1°C$ under crowded conditions. In the rearing room, 30-40% relative humidity was maintained by using humidifiers. The crickets were maintained in white plastic boxes (30 x 40 x 60 cm, H x W x B) covered with netted lids and provided with egg boxes as shelter. During early larval stages, a few thousand crickets were placed in a single box and the number was gradually reduced to a few hundred as they developed into adults. The diet consisted of a 2:4:1 mixture of breeding diet 1310 for rats and mice, breeding diet 2010 for rabbits, and breeding/maintenance diet 5030 for cats provided by Altromin (Lage, Germany). Tap water was supplied *ad libitum* in water containers which were changed three times every week. For reproduction, three times a week, 50 adult female and male crickets (at least 2 to 3 d old) each were put together in a white plastic box, under similar conditions as described above. The boxes were provided with small plastic cups (8 cm in diameter, 3.5 cm high) filled either with tightly packed, fine, moist sand or with moist turf for oviposition. The sand/turf cups were replaced with new cups after 2 to 3 days of egg deposition. In total, eggs were collected from each batch of adult females for about 2 weeks. After egg deposition, fecal particles were removed carefully and four cups were placed in a transparent plastic box (10 x 20 x 20 cm, H x W x B), covered with a transparent plastic lid. From time to time water was added to the cups for keeping the sand/turf moist. After 4 to 5 days, 8 cups were transferred to a white plastic box where the eggs hatched and the crickets were maintained thereafter.

Newly molted larval instar/adult crickets were termed as 0 d old larvae and adults, respectively. Experimental insects of defined age were maintained in separate boxes, and male and female crickets were always separated. If not specifically mentioned, all studies were carried out on 1 d old adult virgin female crickets.

2.1.2. *Schistocerca gregaria*

Locusts, *Schistocerca gregaria* (Caelifera: Acrididae), were bought from b.t.b.e. Insektenzucht and maintained under crowded conditions in a climate chamber until use. The locusts were placed in big cages (45 x 50 x 70 cm, H x W x B) under 14 h light: 10 h dark regime at a constant temperature of $28 \pm 1°C$ and relative humidity of 20-30%, with a provision of 60 W lamps for basking. The locusts were supplied with maize (*Zea mays*) and coleus (*Coleus blumei*) plants in addition to wheat germ *ad libitum*. Newly molted adult locusts were termed as 0 d old adults; males and females were maintained together.

2.2. Chemicals

A23187, No.1234; Biotrend Chemikalien GmbH, Cologne, Germany

$[1-^{14}C]$-Acetic acid, sodium salt, MC-125; Hartmann Analytic, Braunschweig, Germany

Acetonitrile, RotiSolv HPLC-grade; Roth, Karlsruhe, Germany

Adipokinetic hormone, Grybi-AKH, Schgr-AKH II; Bachem Biochemica, Heidelberg, Germany; Pyrap-AKH, kindly provided by Dr. Dalibor Kodrík, Czech Academy of Sciences.

(Note: If not specifically mentioned, Grybi-AKH was used exclusively in all studies.)

Anthrone, A-1631; Sigma, Steinheim, Germany

BAPTA, A-9801; Sigma

BAPTA-AM, A-1076; Sigma

8-Br-cAMP, B-7880; Sigma

BSA, A-3294; Sigma

$^{45}CaCl_2$, NEZ013; PerkinElmer Life Sciences GmbH, Rodgau, Germany

Caffeine 200, No.3309.1; Roth

Cholera toxin, C-8052; Sigma

8-CPT-cAMP, C 010-50E; Biolog Life Science Institute, Bremen, Germany

Cyclic AMP (^3H) assay system, TRK 432; Amersham Biosciences Europe GmbH, Freiburg, Germany

Dantrolene, D-9175; Sigma

Dibutyryl-cAMP, No.1141; Biotrend

DMSO, D-5879; Sigma

EDTA, E-1644; Sigma

EGTA, E-8145; Sigma

Ficoll 400; No.17-0400-01; Amersham

Forskolin, F-6886; Sigma

[^{14}C(U)]-Glycerol, NEC-441X; PerkinElmer

HEPES, H-3375; Sigma

IBMX, I-7018; Sigma

Ionomycin, I-0634, Sigma

Lanthanum chloride, L-4131; Sigma

Medium 199, M-7653; Sigma

Methanol, HPLC-grade; Roth

MOPS, M-5162; Sigma

Nifedipine, N-7634; Sigma

Oleic acid, O-1630; Sigma

[9,10-^{3}H]-Palmitic acid, MT-845; Hartmann

Palmitic acid, P-0500; Sigma

PMA, No.1201; Biotrend

Quin 2-AM, Q-4875; Sigma

Roti-Nanoquant, K880.1; Roth

Roti-Quant, K015.1; Roth

Ruthenium red, R-2751; Sigma

Ryanodine, R-6017; Sigma

Scintillation cocktails: a. Rotiszint 11, No.8005.1; Roth

                        b. Lumasafe Plus, Lamac LSC B.V., No.3097; Groningen, The Netherlands

Sp-5,6-DCI-cBIMPS, D014-05; Biolog

Suramin, No.1472; Biotrend

Thapsigargin, No.1138; Biotrend

Theophylline, T-1633; Sigma

TMB-8 HCl, T-111; Sigma

Tricine, T-9784; Sigma

Triolein , T-7140; Sigma

[carboxyl-^{14}C]-Triolein, NEC-674; PerkinElmer

Triton X-100, X-100; Sigma

Vanillin, No.94750; Fluka, Neu-Ulm, Germany

(±)-Verapamil hydrochloride, V-4629; Sigma

W-7 hydrochloride, No.0369; Biotrend

Water for chromatography, No.1.15333.2500; Merck, Darmstadt, Germany

If not mentioned, all other chemicals were of p.A. quality from Merck and Sigma.

2.3. Instruments

Cleanbench Lamin Air HBB 2448; Heraeus Instruments, Hanau, Germany

Centrifuges: a. Biofuge 13; Heraeus Sepatech GmbH, Osterode , Germany

          b. Sigma 3K12; Sigma Laborzentrifugen, Osterode, Germany

Hamilton Syringe, Nr. 710 SNCH, pst 4, 33-gauge; Hamilton AG, Bonaduz, Switzerland

Hand-held homogenizer, No.749540-0000; Kontes Glass Company, Vineland, New Jersey, USA

Incubator, BK 6160; Heraeus Instruments

Liquid Scintillation Counter (LSC) Tri-Carb 2100 TR; Canberra-Packard GmbH, Dreieich, Germany

Magnetic stirrer, IKAMAG RCT basic; Janke & Kunkel GmbH & Co. KG, Staufen, Germany

Speed-Vac-Concentrator, Alpha RVC; Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany

Spectrophotometer, Ultraspec III; Pharmacia LKB, Freiburg, Germany

Ultrasonic bath, Transsonic 310; Elma, Singen/Htwl., Germany

2.4. Miscellaneous

Computer programs: a. Statistics - WinStat version 3

            b. Graphs - SigmaPlot 7

Micropipettes, No.709103; Brand, Wertheim, Germany

Syringe filter, No.17597K; Sartorius AG, Göttingen, Germany

Sand, 0.1 - 0.5 mm particle size; BayWa AG, Bindlach, Germany

TLC plates: a. SIL G-25, 20 x 20 cm, K798.1; Roth

        b. Silica gel 60 F_{254}, No.1.05715; Merck

Turf; Hagebaumarkt, Bayreuth, Germany

2.5. Incubation media for fat body *in vitro* studies

2.5.1. Modified Medium 199 (MM199)

Medium 199 was fortified with 25 mM HEPES and 1% Ficoll 400. Calcium chloride and sodium acetate were added to final concentrations of 3 and 10 mM respectively, pH was adjusted to 7.2. The medium was sterilized by filtering through 0.2 µm cellulose acetate filter and stored at 2°C.

2.5.2. Fat body incubation medium (FBIM)

Chemical	Final concentration
Na-acetate.3H$_2$O	10 mM
NaCl	137 mM
KCl	6 mM
MgSO$_4$.7H$_2$O	2 mM
K$_2$HPO$_4$	1 mM
glucose	10 mM
sucrose	50 mM
BSA (Fraction V)	2%
HEPES	25 mM
CaCl$_2$.2H$_2$O	3 mM

pH was adjusted to 7.2 and the medium was filter-sterilized and stored at 2°C. For some experiments CaCl$_2$.2H$_2$O was replaced by 1 mM EGTA (referred to as FBIM+EGTA).

2.6. Fat body dissection and incubation *in vitro*

Total abdominal fat bodies, in the case of larval instars, and free abdominal fat bodies, in the case of adults, were dissected. The fat body could be divided into halves or quarters without loss of lipogenic activity (Lorenz, 2001). Fat body pieces dissected from the experimental insects were rinsed with MM199/FBIM and incubated in the same medium with the substance under investigation at 27°C under humid conditions with shaking. If not specifically mentioned, all incubations were carried out for 45 min. In some experiments preincubations were carried out under similar conditions for shorter durations (10-30 min). At the end of preincubation the fat bodies were transferred to the medium prepared for actual incubation. Incubations longer than 1 h were carried out under oxygen-enriched conditions. Controls were incubated in medium containing the carrier of the substance only.

2.7. Enzyme assays

2.7.1. Acetyl-CoA carboxylase (ACCase; EC 6.4.1.2)

2.7.1.1. Enzyme preparation

Fat bodies were rinsed with homogenization buffer (HB; 0.5 M glycerol, 2 mM EDTA, 5 mM DTT, and 0.32 mM PMSF in 0.1 M HEPES-KOH buffer, pH 7.5) and homogenized in 150 µl HB using a hand-held homogenizer. The homogenate was centrifuged at 4°C for 10 min at 20,000g, to get infranatant covered with a floating fat layer. The infranatant was transferred to a new tube with a gel-loading tip. The infranatant was recentrifuged and the resulting infranatant was collected in a new tube (enzyme preparation). Unless otherwise mentioned, all steps were carried out at 4°C.

2.7.1.2. Enzyme assay

The enzyme preparation was spectrophotometrically assayed for ACCase activity by coupling the production of ADP to the oxidation of NADH by pyruvate kinase and lactic dehydrogenase following Evenson et al. (1994). The reaction mixture contained 0.4 M glycerol, 50 mM KCl, 0.5 mM DTT, 5 mM $MgSO_4$, 0.1 M Tricine-KOH buffer, pH 7.4, 2.5 mM ATP, 15 mM $NaHCO_3$, 0.32 mM NADH, 0.5 mM phosphoenolpyruvate, 1.4 units of pyruvate kinase, 2 units of lactic dehydrogenase, the enzyme preparation, and 0.5 mM acetyl-CoA in a final volume of 0.5 ml. The reaction was initiated by adding acetyl-CoA and the changes in the optical density were recorded at 340 nm at RT. The rate of NADPH oxidation prior to acetyl-CoA addition served as a blank value which was subtracted from the overall rate. One unit of enzyme activity is defined as the amount of enzyme required to catalyze the carboxylation of 1 µmol acetyl-CoA per min under the conditions described above.

2.7.2. ATP:citrate lyase (ACL; EC 2.3.3.8)

Enzyme preparation was carried out, as described for ACCase, by using 0.25 M sucrose in 20 mM Tris-HCl buffer, pH 7.1 as a HB. The enzyme preparation was spectrophotometrically assayed for ACL activity, according to Srere (1959), with a few modifications. Acetyl-CoA and oxaloacetate are two of the products formed when citrate is incubated with ATP, CoA, Mg^{++} and ACL. Oxaloacetate reacts with NADH in the presence of malic dehydrogenase and the decrease in absorbance at 340 nm was used to determine the enzyme concentration. The assay mixture contained 100 mM Tris-HCl

buffer, pH 9.2, 10 mM $MgCl_2$, 10 mM dithioglycerol, 31.5 mM tripotassium citrate, 6 units of malic dehydrogenase, 0.3 mM CoA, 0.2 mM NADH, 5 mM ATP, and the enzyme preparation in a final volume of 0.5 ml. The reaction was initiated by adding ATP and the changes in the optical density were recorded at 340 nm at RT against a blank cell containing all components except NADH and CoA. ACL activity was directly proportional to the concentration of citrate in the reaction mixture. One unit of enzyme activity is defined as the amount of enzyme required to oxidize 1 µmol NADH per min under the assay conditions.

2.7.3. Fatty-acid synthase (FAS; EC 2.3.1.85)

Enzyme preparation was carried out, as described for the ACCase, by using 1 mM DTT and 1 mM EDTA in 50 mM potassium phosphate buffer, pH 7.4 as a HB. The enzyme preparation was spectrophotometrically assayed for FAS activity, following Arslanian and Wakil (1975), based on oxidation of NADPH. The reaction mixture contained 100 mM KPO_4 buffer, pH 6.9, 5 mM DTT, 150 µM NADPH, 25 µM acetyl-CoA, 250 µM malonyl-CoA and the enzyme preparation in a final volume of 0.5 ml. The reaction was initiated by adding malonyl-CoA and the changes in the optical density were recorded at 340 nm at RT. The rate of NADPH oxidation prior to malonyl-CoA addition served as a blank value which was subtracted from the overall rate. FAS showed highest activity at pH 6.9 when tested over a pH range of 6.5 to 7.8. One unit of enzyme activity is defined as the amount of enzyme required to oxidize 1 nmol of NADPH per min under the assay conditions.

2.7.4. Triacylglycerol lipase (TGL; EC 3.1.1.3)

2.7.4.1. Radiolabeled substrate

[carboxyl-^{14}C]-Triolein, with a specific activity of 3.8×10^3 MBq/mmol was supplied as 3.7 MBq/ml in toluene: ethanol, 1:1. Upon TLC analysis, radiolabeled triolein was found to be more than 99% pure, hence it was used without further purification. The solvent was evaporated under a flow of nitrogen and the radiolabeled triolein was taken up in ethanol containing 130 mM Triton X-100. To this, non-radiolabeled triolein was added to obtain a final concentration of 1 mM triolein with a final specific activity of 100 MBq/mmol (radiolabeled substrate). The radiolabeled substrate could be stored at -70°C for up to several months before use. A range of Triton X-100 concentrations from 32.5 mM up to 260 mM was tested and a concentration of 130 mM, i.e. a final concentration of 13 mM in the reaction mixture, was found to be optimum for TGL activity.

2.7.4.2. Enzyme assay

Enzyme preparation was carried out, as described for the ACCase, using 0.25 mM EDTA in 10 mM Tris-HCl buffer, pH 7.4 as a HB. The enzyme preparation was assayed for TGL activity according to Hirayama and Chino (1987), with some modifications. This radiochemical assay is based on the release of free fatty acids from radiolabeled triolein by the action of TGL. The radiolabeled substrate (25 µl) was delivered into 2 ml Eppendorf tubes and the solvent was evaporated under a flow of nitrogen to leave behind triolein-Triton X-100 droplets. To this, 225 µl assay buffer (20 mM MOPS-NaOH, pH 6.9) was added and mixed very well. The reaction was started by the addition of 25 µl of enzyme preparation and the tubes were incubated at 27°C for 45 min with shaking. The incubation was stopped by adding 1.25 ml fatty acid extraction solution (0.5 µM oleic acid in chloroform: methanol: benzene, 2:2.4:1) and 25 µl 1 N NaOH. After vigorously vortexing, the tubes were centrifuged at RT for 10 min at 20,000g. From the upper aqueous phase containing free fatty acids, a 200 µl fraction was mixed with 2.5 ml Lumasafe Plus scintillation cocktail for counting radioactivity. One unit of enzyme activity is defined as the amount of enzyme required to release 1 nmol fatty acid per h under the assay conditions. The activity of TGL was linear over a final concentration range of 0.025 to 0.2 mM of triolein in the reaction mixture. Upon freezing and thawing TGL lost activity to a small extent.

2.8. Glycogen and free carbohydrate estimation

Glycogen and free carbohydrate were estimated by the anthrone method (Mokrasch, 1954), with a few changes. Samples were heated with 100 µl of concentrated sulfuric acid and 1 ml of anthrone reagent (0.13% anthrone in 67% sulfuric acid) at 90°C for 10 min. After cooling, absorbance was measured at 585 nm against a reference containing only sulfuric acid and anthrone reagent. A standard graph of 0 to 20 µg glycogen against A_{585} was plotted, to determine glycogen or free carbohydrate concentrations.

2.9. Lipid estimation

Lipid was estimated by the sulphophosphovanillin method according to Zöllner and Kirsch (1962), with a few modifications. Samples were heated with 100 µl concentrated sulfuric acid at 100°C for 10 min. After cooling, 1 ml of 0.2% phosphovanillin in 57% ortho-phosphoric acid was added and the tubes were put in dark for 20 min. Absorbance was measured at 530 nm against a reference containing only sulfuric acid and phosphovanillin reagent. A standard graph of 0 to 30 µg cholesterol against A_{530} was plotted, to determine lipid concentrations.

2.10. Protein estimation

Protein was estimated by Roti-Nanoquant or Roti-Quant solution based on modified Bradford's protein assay.

2.10.1. By Roti-Nanoquant

Protein samples in a final volume of 200 µl were mixed with 750 µl Roti-Nanoquant working solution. Immediately, absorbance was measured at 590 and 450 nm against water. A standard graph of 0 to 20 µg BSA was plotted against the quotient of A_{590}/A_{450}, to determine protein concentrations.

2.10.2. By Roti-Quant

Protein samples in a final volume of 750 µl were mixed with 250 µl Roti-Quant solution. Within 5 - 30 min, absorbance was measured at 595 nm against 750 µl water mixed with 250 µl Roti-Quant solution. A standard graph of 0 to 40 µg BSA was plotted against A_{595}, to determine protein concentrations.

2.11. Extraction of fat body lipid, protein, free carbohydrate and glycogen

Fat bodies were dissected and transferred into pre-weighed 1.5 ml safe-lock Eppendorf tubes, containing 20 mg of Na_2SO_4 and 200 µl of 75% methanol in water. The procedure used for the extraction and separation of fat body lipid, protein, glycogen and free carbohydrate was as shown in Fig. 1. The efficiency and the recovery rates of the extraction and separation were checked using standard samples containing either only lipid, protein, glycogen, or glucose, respectively, or combinations thereof. Blanks (containing 20 mg of Na_2SO_4 and 200 µl of 75% methanol in water only) and two internal standards (one containing 4 mg of cholesterol, 1 mg of BSA, and 100 µg of glycogen, the other additionally containing 100 µg of glucose) were carried through each experiment to monitor the efficiency of extraction and of the separation into substance classes. High extraction efficiencies for lipid ($91.1 \pm 2.1\%$), protein ($72.7 \pm 2.6\%$), glycogen ($83.4 \pm 1.9\%$) and free carbohydrate ($96.5 \pm 2.4\%$) ($n = 40\text{-}50$) were achieved. Glycogen and free carbohydrate were separated effectively with virtually no cross-contamination. The results obtained for the samples were corrected for recovery rates and the sample blanks.

put sample into pre-weighed Eppendorf tube, containing 20 mg Na_2SO_4 and 200 µl of 75% MeOH in H_2O to determine sample fresh-weight
homogenize sample using a hand-held motor pestle
rinse pestle with 600 µl $CHCl_3$:MeOH (1:1) directly into the Eppendorf-tube, vortex and centrifuge

supernatant 1
remove supernatant carefully, transfer into 2 ml Eppendorf tube

pellet
add 300 µl $CHCl_3$:MeOH (1:1), vortex, put in ultrasonic bath, vortex, centrifuge

supernatant 2
remove supernatant carefully, combine with supernatant 1

pellet
dry in thermoblock at 70°C, add 500 µl of Na_2SO_4-saturated 66% EtOH, vortex, put in ultrasonic bath, vortex to dissolve pellet completely, centrifuge

to the combined supernatants, add 500 µl of $CHCl_3$ and 300 µl of 1M NaCl, vortex, centrifuge

remove supernatant quantitatively, discard

remove aqueous epiphase quantitatively, dry epiphase in Speed-Vac, redissolve resulting precipitate in 500 µl of H_2O, take aliquot for determination of **free carbohydrate**

pellet
dry pellet completely in thermoblock at 70°C, add 200 µl of 10% KOH, vortex, put in ultrasonic bath, vortex, macerate for 30 min at 100°C in a thermoblock, vortex every 10 min

dry organic hypophase in Speed-Vac, redissolve resulting precipitate in 1000 µl of hexane, add 500 µl of 1 M NaCl, vortex, centrifuge, take aliquot for determination of **lipid**

take a 50 µl aliquot and mix with 150 µl of H_2O, take aliquot for the determination of **protein**

add 150 µl of EtOH, vortex, leave standing for 10 min, centrifuge

remove supernatant quantitatively, discard

pellet
dry pellet completely in thermoblock at 70°C, redissolve in 400 µl of H_2O at 100°C, take aliquot for determination of **glycogen**
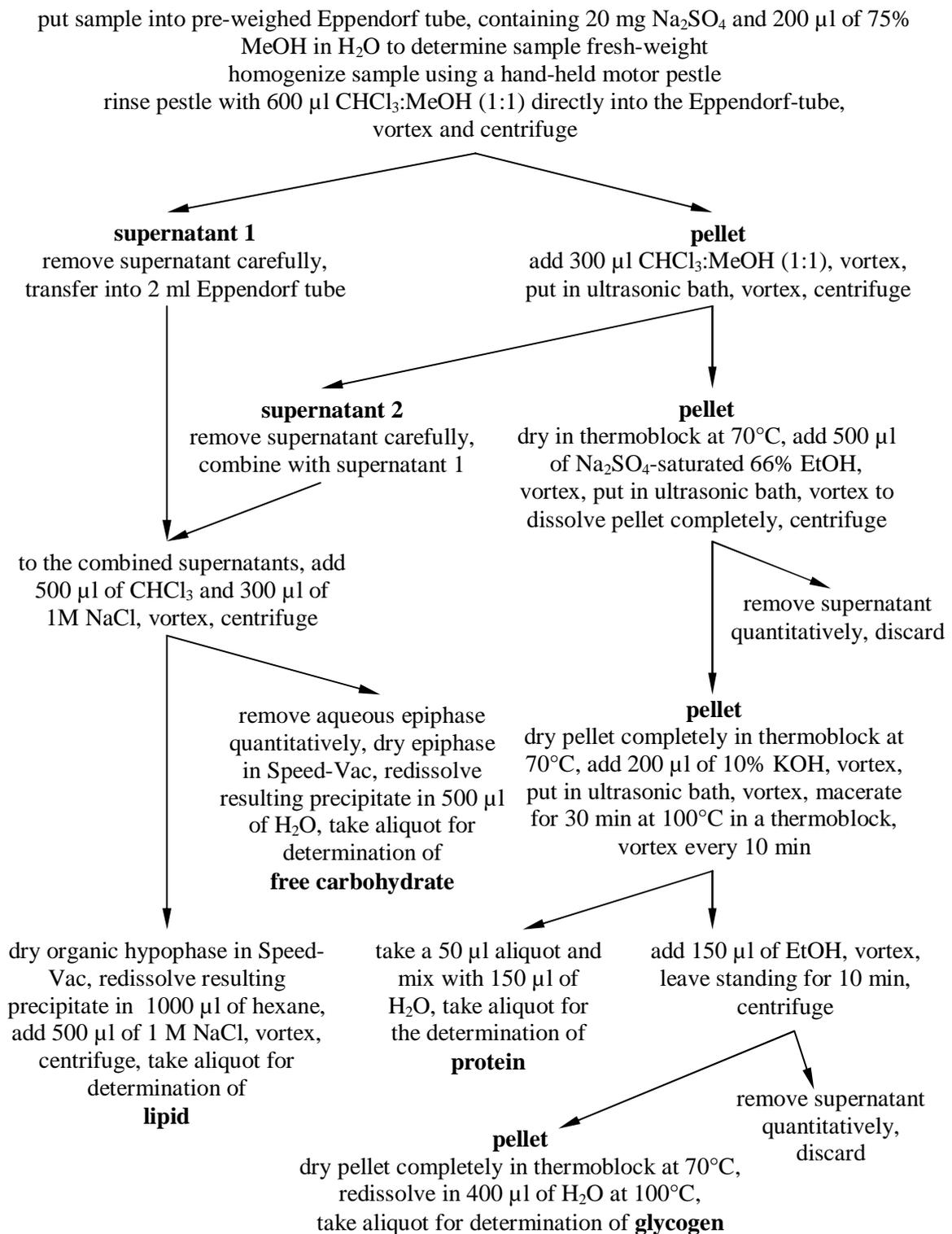
Fig. 1: The flow scheme showing the method used for the extraction and separation of organic substances from fat bodies was taken from Lorenz (2003). The centrifugations were performed for 10 min each at 4°C and 21,000g. The ultrasonic bath treatments were for 5 min each.

2.12. Topical application of AKH

Two µl of hemolymph was collected from 3 d old adult female crickets, by pricking with a fine needle at the base of a metathoracic leg. This was mixed with 100 µl concentrated sulfuric acid, for the estimation of lipid. Immediately thereafter, 100 pmol AKH dissolved in 2 µl 20% DMSO was topically applied onto the dorsal thorax under wings. The insects were maintained individually for 2.5 h with food and water freely available. After this time, 2 µl hemolymph was collected again for lipid estimation as described above and the fat bodies were dissected. After rinsing with HB, the fat bodies were homogenized in 150 µl HB and assayed for TGL activity. The control insects were topically applied with 2 µl of 20% DMSO only.

2.13. Radiolabeled precursor incorporation into lipid by fat body *in vitro*

2.13.1. Acetate incorporation

The assay for acetate incorporation into lipid was carried out according to Lorenz (2001), with a few changes. The fat bodies were incubated in 95 µl radiolabeled MM199 (1.7 MBq [1-^{14}C]Na-acetate per mmol) in 2 ml Eppendorf tubes at 27°C for 60 min under humid conditions with continuous shaking. The incubation was stopped by the addition of 200 µl ice cold methanol followed by 500 µl isooctane and 500 µl 1 M NaCl. After vortexing, the tubes were put on ice for 30 min. The tubes were vortexed once more and sonified in ice cold ultrasonic bath for 5 min followed by vortexing and centrifugation at 4°C for 5 min at 20,000g. From the upper organic phase a 200 µl aliquot was pipetted into 2.5 ml scintillation cocktail, Rotiszint 11, for the determination of radioactivity incorporated into lipid. For the blank value, the incubation and extraction were carried out without fat body.

2.13.2. Glycerol incorporation

For the determination of glycerol incorporation into lipid, essentially the same procedure as described for acetate incorporation was followed with a single change, i.e. instead of [1-^{14}C]Na-acetate, [^{14}C(U)]-glycerol was used as a radiolabel. [^{14}C(U)]-Glycerol (specific activity 5.3 GBq per mmol in ethanol: H$_2$O, 1:1) was delivered in a tube and the solvent was evaporated under a flow of nitrogen. The radioactivity was taken up in the MM199 (additionally containing 2 mM non-radioactive glycerol) to give a final specific activity of 16.6 MBq/mmol.

2.13.3. Palmitate incorporation

For the determination of palmitate incorporation into lipid, essentially the same procedure as described for acetate incorporation was followed with a few changes. Instead of [1-^{14}C]Na-acetate, [9,10-^{3}H]-palmitic acid was used as a radiolabel in the incubation medium. The radiolabeled palmitic acid supplied as 37 MBq/ml (specific activity 1.85 TBq/mmol) in ethanol was delivered in a tube and the solvent was evaporated under a flow of nitrogen. To this, non-radioactive palmitic acid (dissolved in ethanol containing 130 mM Triton X-100), and MM199 were added to obtain a final concentration of 0.1 mM palmitic acid with a final specific activity of 2.03 GBq/mmol. Final concentrations of ethanol and Triton X-100 in the incubation medium were 0.1% (v/v) and 0.13 mM respectively. At the end of incubation, fat bodies were rinsed with MM199 and lipids were extracted for the determination of radioactivity incorporated into lipid as described for acetate incorporation assay. The extracted lipids were also used for detailed analysis by TLC.

2.14. Reversibility of acetate incorporation inhibition

Caffeine strongly inhibited acetate incorporation by fat body *in vitro*. The reversibility of this effect was studied as follows. The fat bodies were incubated with FBIM containing 50 mM caffeine for 1 h. At the end of the first incubation, the medium was pipetted off, FBIM without caffeine was added and the fat bodies were further incubated for 1 h. The fat bodies were then transferred to [1-^{14}C]Na-acetate labeled FBIM and incubated for 30 min to determine acetate incorporation activity. Two control sets were run in parallel. The first control set was incubated in FBIM without caffeine, while the second control set was incubated in FBIM added with 50 mM caffeine throughout the experiment.

Theophylline, thapsigargin and ionomycin also strongly inhibited acetate incorporation by fat bodies. The reversibility of their inhibitory effects was studied using the method used for caffeine, replacing caffeine with either by 32 mM theophylline or 50 µM thapsigargin or 50 µM ionomycin. The correct amounts of DMSO (solvent for thapsigargin) or ethanol (solvent for ionomycin) were added to FBIM whenever thapsigargin or ionomycin was not included in the corresponding medium. Caffeine and theophylline were directly dissolved in FBIM to the final concentrations mentioned above.

2.15. Influx and efflux studies

2.15.1. Influx of acetate and calcium

Fat bodies were dissected from adult female crickets and rinsed in MM199 followed by a preincubation in the same medium at 27°C for 20 min under humid conditions with shaking. The fat bodies were then incubated in 100 µl MM199 containing AKH and labeled either with [1-^{14}C]Na-acetate (1.7 MBq/mmol) or ^{45}CaCl$_2$ (110.1 MBq/mmol) for a short period of time (20 sec to 4 min). At the end of the incubation, the fat bodies were rinsed four times with 500 µl MM199 each to remove attached radiolabel. The fat bodies were then extracted with 200 µl methanol, 500 µl isooctane and 600 µl 1 M NaCl. After vortexing, the tubes were put on ice for 30 min. The tubes were vortexed once more and sonified in ice cold ultrasonic bath for 5 min followed by vortexing and centrifugation at 4°C for 5 min at 20,000g. From the upper organic phase a 300 µl aliquot was pipetted into 2.5 ml Rotiszint 11 for the determination of radioactivity incorporated into lipid. From the lower aqueous phase a 400 µl aliquot was pipetted into 2.5 ml Lumasafe Plus for the determination of ^{14}C-acetate or ^{45}Ca^{2+}. In the case of control fat bodies, all the incubations were carried out without AKH with the rest of the procedure similar to the AKH-treated fat bodies.

2.15.2. Efflux of calcium

Fat bodies were dissected from adult female crickets and rinsed in MM199. The fat bodies were prelabeled with ^{45}CaCl$_2$ by incubating in 100 µl MM199 containing ^{45}CaCl$_2$ (110.1 MBq/mmol) at 27°C for 75 min under humid conditions with shaking. After prelabeling, the fat bodies were rinsed four times with MM199 to remove attached radiolabel. The fat bodies were then transferred to 200 µl MM199 and incubated for a short period (Efflux1; 30 sec to 4 min) with very gentle vortexing throughout. After removing the fat bodies, an aliquot of 100 µl was pipetted from the medium and mixed with 2.5 ml Lumasafe Plus, to determine ^{45}Ca^{2+} released during Efflux1. The fat bodies were once more transferred to ^{45}CaCl$_2$ labeled MM199 for relabeling with ^{45}CaCl$_2$, at 27°C for 40 min. They were rinsed four times and transferred to 200 µl MM199 containing AKH and incubated for a short period (Efflux2; 30 sec to 4 min) with very gentle vortexing throughout. At the end of incubation the fat bodies were removed and an aliquot of 100 µl was pipetted from the medium and mixed with 2.5 ml Lumasafe Plus to determine ^{45}Ca^{2+} released during Efflux2. The fat bodies were extracted as described for the influx studies and the

radioactivity present in organic and aqueous phases was determined. The comparison of $^{45}Ca^{2+}$ released during Efflux1 and Efflux2 was used to determine the effect of AKH on $^{45}Ca^{2+}$ efflux from the fat body. For the control fat bodies both Efflux1 and Efflux2 were studied by incubation in medium without AKH.

2.16. TLC

Lipids extracted from the fat bodies, directly or after saponification, were spotted on a 20 x 20 cm TLC-plate. Lipid standards were spotted along with the samples and the plate was developed with 1,2-dichloroethane: methanol: acetic acid (for Silica gel 60 F_{254}- 93.5: 6.5: 0.4, and for SIL G-25- 97.5: 2.5: 0.1, v: v: v) in a saturated atmosphere for approximately 50 min. The plate was dried under a flow of hot air, and to visualize lipids, 10% phosphomolybdic acid in ethanol was sprayed onto the plate, followed by heating at 200°C for 3 min. The lanes with radioactive samples were covered with glass plates while spraying the phosphomolybdic reagent. The gel bands in the radioactive sample lanes were scraped off and collected in scintillation vials containing 3 ml Lumasafe Plus, for the determination of radioactivity present.

2.17. Saponification of extracted lipid samples

A 20 µl aliquot of extracted lipid samples (organic phase) was delivered into an Eppendorf tube containing 500 µl methanol. The organic solvent was evaporated under a gentle stream of nitrogen. To this 100 µl of 30% KOH was added and incubated at 70°C for 60 min. Then 50 µl concentrated HCl and 200 µl isooctane were added. The tubes were vortexed and sonicated in ice cold water bath for 5 min. The tubes were vortexed again and centrifuged at 4°C for 5 min at 20,000g to achieve phase separation. From the upper organic and lower aqueous phase aliquots were pipetted into 2.5 ml scintillation cocktail, Rotiszint 11 and Lumasafe Plus, respectively for the determination of radioactivity present. Additional aliquots from both the phases were spotted on TLC plate for analysis.

2.18. cAMP measurement

A fat body was homogenized in 150 µl 0.05 M Tris buffer containing 4 mM EDTA. To coagulate proteins, the homogenate was heated on a heating block at 100°C for 10 min and cooled on ice for 5 min. The sample was centrifuged at 4°C for 10 min at $20,000g$ and the infranatant was transferred to a new tube. The infranatant was recentrifuged, the resulting infranatant was collected in a new tube and an aliquot was assayed for cAMP content using a commercially available competitive binding protein assay (Amersham Biosciences). In addition, soluble protein was estimated.

2.19. Statistical analysis

All results are presented as mean values ± S.E. and were compared by the Mann-Whitney U-test. The age-dependency of the changes was tested using the Spearman rank-correlation. The correlations between time series were calculated using Pearson cross-correlation.

3. Results

3.1. Regulation of energy metabolism by AKH

3.1.1. Effect of AKH on lipid synthesis

3.1.1.1. Comparison between MM199 and FBIM

The rate of acetate incorporation into lipid was higher when the fat bodies were incubated in MM199 compared to FBIM, but the difference was not significant. Addition of 1% Ficoll 400 to FBIM further reduced the acetate incorporation activity by about 10%, and therefore, Ficoll 400 was not included in FBIM even though it was included in MM199. Acetate incorporation by fat bodies was significantly inhibited by 10^{-8} M AKH in adult female crickets (Fig. 2). The inhibitory effect of AKH on lipogenesis was slightly lowered in FBIM. Since M199, the basic medium used to prepare MM199, contains calcium and acetate, FBIM was used in the experiments where these components were to be manipulated or excluded. AKH from *Pyrrhocoris apterus* (Pyrap-AKH) also significantly inhibited acetate incorporation by adult cricket fat bodies ($84.9 \pm 2.4\%$ inhibition at 10^{-9} M AKH concentration).
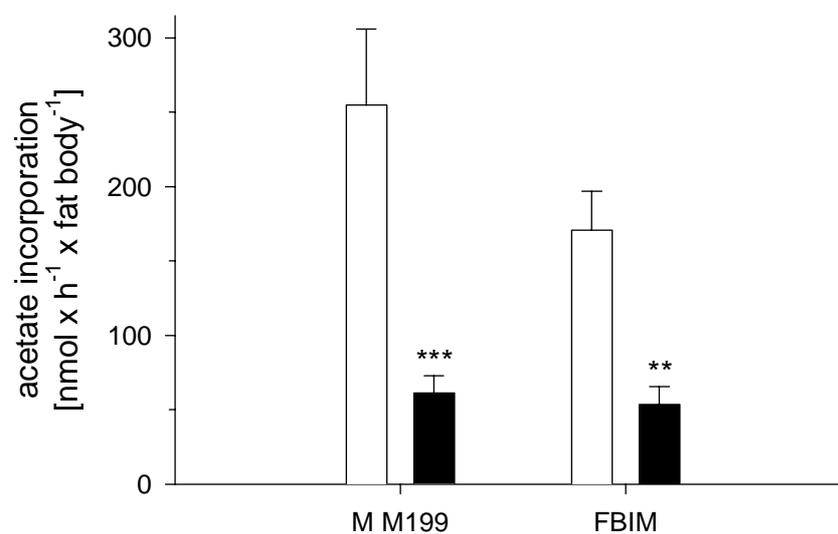

Fig. 2: Inhibition of acetate incorporation by 10^{-8} M AKH in adult female cricket fat bodies *in vitro*. Control (white bars) and AKH-treated (black bars) fat bodies were incubated either in MM199 or FBIM. All results presented here correspond to the activity per whole fat body from an insect. Means $\pm$ S.E. of 10 determinations; **, $P < 0.01$; ***, $P < 0.001$.

3.1.1.2. Effects of AKH, glycerol and palmitate on acetate incorporation

The effects of AKH, glycerol and palmitate on acetate incorporation by fat bodies in adult female crickets were studied in the incubation medium MM199. Acetate incorporation was not affected by glycerol, either independently or in combination with AKH. In the presence of palmitate, at higher concentrations, there was a tendency for reduced incorporation, but the effect was not significant (Table 1).

Table 1:   Effects of AKH, glycerol and palmitate on acetate incorporation[§]

Treatment	Acetate incorporation [nmol/h/fat body]	
	Control	Test
1 mM glycerol	109.5 ± 8.2	130.9 ± 11.0
5 mM glycerol	109.5 ± 8.2	140.8 ± 15.9
10 mM glycerol	109.5 ± 8.2	106.0 ± 7.9
10^{-10} M AKH	109.5 ± 8.2	38.8 ± 5.0***
10 mM glycerol + 10^{-10} M AKH	109.5 ± 8.2	33.9 ± 4.3***
0.01 mM palmitate	65.1 ± 4.9	70.1 ± 6.4
0.1 mM palmitate	65.1 ± 4.9	53.8 ± 4.3
0.5 mM palmitate	65.1 ± 4.9	38.9 ± 5.2

[§]Acetate incorporation activity in adult female cricket fat bodies in the presence of AKH, glycerol and palmitate *in vitro*. The asterisks indicate significant change in activity brought about by the treatment as determined by the Mann-Whitney U-test. Means $\pm$ S.E. of 40 to 100 determinations; ***, $P < 0.001$.

3.1.1.3. Effects of AKH and palmitate on glycerol incorporation

A several-fold increase in glycerol incorporation into lipid by fat bodies was observed upon AKH treatment, while 0.1 mM palmitate had no significant effect (Table 2). Fat bodies from adult female crickets were treated with 10^{-8} M AKH in the incubation medium MM199, containing either 0.61 mM or 10 mM acetate. Higher acetate concentrations augmented the effect of AKH. A similar increase was observed when fat bodies from 9-11 d old adult female *Schistocerca gregaria* were treated with 10^{-7} M AKH.

Table 2:   Effects of AKH and palmitate on glycerol incorporation activity[§]

Insect	0.61 mM acetate		10 mM acetate	
	Control	Test	Control	Test
		10^{-8} M AKH		10^{-8} M AKH
G. bimaculatus	9.0 ± 1.3	47.2 ± 9.8**	8.5 ± 1.7	62.4 ± 14.0***
		0.1 mM palmitate		0.1 mM palmitate
	4.2 ± 1.1	2.3 ± 0.4	4.4 ± 0.9	3.1 ± 0.6
				10^{-7} M Schgr-AKH II
S. gregaria	−	−	14.4 ± 5.4	61.7 ± 19.9**

[§]Effects of AKH and palmitate on glycerol incorporation activity *in vitro*. Rate of glycerol incorporation expressed in nmol/h/fat body. Means ± S.E. of 10 to 20 determinations; **, $P < 0.01$; ***, $P < 0.001$.

3.1.1.3.1. TLC analysis

The effect of AKH on glycerol incorporation into different lipid classes by the adult female cricket fat body was analyzed by TLC. An aliquot of organic phase, containing lipids extracted from the fat bodies incubated in MM199 labeled with [^{14}C(U)]-glycerol, was spotted on a TLC-plate. After separation, the majority of the radioactivity was recovered in a TAG fraction (Table 3). The distribution of radioactivity into different lipid classes was similar in the controls and in the fat bodies treated with 10^{-8} M AKH.

Table 3:   Effect of AKH on glycerol incorporation into lipid classes[§]

Class	% radioactivity	
	Control	Test
TAG	81.0	77.4
1,2-DAG	7.3	12.5
Cholesterol + FA	2.5	1.8
Rest	9.2	8.3

[§]The distribution of radioactivity into different lipid classes in the control and 10^{-8} M AKH-treated fat bodies, presented as % radioactivity recovered. The fat bodies were incubated with AKH for 3 h. Values are means of two independent experiments.

A second aliquot of the organic phase was saponified. It showed that, in the case of AKH-treated samples, 94.8% of the radioactivity was present in the aqueous phase (76.3% in the case of controls). The rest of the radioactivity, present in the organic phase, was due to unsaponified lipid, as confirmed by TLC. The aqueous phase, containing most of the radioactivity, was also analyzed by TLC. In both AKH-treated and control samples, almost all radioactivity (about 96%) was recovered at the starting line, which suggests that it was present in the form of glycerol only.

3.1.1.4. Effect of AKH on palmitate incorporation

The effect of 10^{-8} M AKH on palmitate incorporation into lipid by adult female cricket fat bodies was studied in the incubation medium MM199. AKH inhibited palmitate incorporation by only $13.8 \pm 2.4\%$ (P = 0.016, n = 5) compared to control fat bodies which incorporated 3.90 ± 0.13 nmol palmitate/h/fat body.

3.1.1.4.1. TLC analysis

The effects of AKH on palmitate incorporation into different lipid classes by the adult female cricket fat body were analyzed by TLC. An aliquot of organic phase containing lipids extracted from the fat bodies incubated in MM199 labeled with $[9,10-^3H]$-palmitic acid was spotted onto a TLC-plate. After separation, the radioactivity was recovered in many different fractions (Table 4). The distribution of radioactivity into different lipid classes was comparable in the controls and in the fat bodies treated with 10^{-8} M AKH.

Table 4:   Effect of AKH on palmitate incorporation into lipid classes[§]

Class	% radioactivity	
	Control	Test
1,2-DAG	19.9	26.5
TAG	15.4	15.2
PL	15.2	8.7
MAG	14.5	12.5
FA	12.8	14.1
Cholesterol	7.3	10.1
Rest	14.9	12.9

[§]The distribution of radioactivity into different lipid classes in the control and 10^{-8} M AKH treated fat bodies, presented as % radioactivity recovered. The fat bodies were incubated with AKH for 2 h. Values are means of three independent experiments.

3.1.2. Effect of AKH on enzymes of lipid metabolism

3.1.2.1. Acetyl-CoA carboxylase

ACCase catalyzes the carboxylation of acetyl-CoA to malonyl-CoA, which is the first committed step in fatty acid synthesis. Fat bodies from 2-4 d old adult female crickets were treated with 10^{-6} M AKH *in vitro*, in the incubation medium MM199. No significant effect of AKH on ACCase activity was observed (Table 5).

Table 5:   Effect of AKH treatment on fat body ACCase activity *in vitro*[§]

Age [d]	Control	Test
		10^{-6} M AKH
2	18.0 ± 2.5	19.5 ± 1.1
3	14.6 ± 1.0	14.8 ± 1.3
4	13.7 ± 1.2	17.2 ± 2.0

[§]Age expressed in days and ACCase activity expressed in nmol/min/mg protein. Means $\pm$ S.E. of 6 to 30 determinations.

3.1.2.2. ATP:citrate lyase

ACL is one of the key enzymes of lipogenesis, responsible for production of cytoplasmic acetyl-CoA. Fat bodies from 1-3 d old adult female crickets were treated with 10^{-8} to 10^{-6} M AKH *in vitro*, in the incubation medium MM199. No significant effect of AKH on ACL activity was observed. Addition of AKH to the HB also had no effect on ACL activity (Table 6).

Table 6:   Effect of AKH treatment on fat body ACL activity *in vitro*[§]

Age [d]	Control	Test
		10^{-8} M AKH
2	31.1 ± 1.5	34.6 ± 3.1
		10^{-7} M AKH
1	44.9 ± 5.0	44.2 ± 5.9
2	54.1 ± 4.4	43.9 ± 5.3
3	45.3 ± 5.9	49.2 ± 9.8
		10^{-6} M AKH
1	45.5 ± 3.6	39.3 ± 4.0
2	41.1 ± 3.0	46.7 ± 3.9

[§]Age expressed in days and ACL activity expressed in µmol/min/mg protein. Means ± S.E. of 4 to 12 determinations.

3.1.2.3. Fatty-acid synthase

Fat bodies from 0-4 d old adult female crickets were treated with 10^{-7} and 10^{-6} M AKH *in vitro* in the incubation medium MM199. No consistently significant effect of AKH on FAS activity was observed, even after addition of AKH to the HB (Table 7).

Table 7:   Effect of AKH treatment on fat body FAS activity *in vitro*[§]

Age [d]	Control	Test
		10^{-7} M AKH
1	74.5 ± 10.2	86.8 ± 10.0
2	78.1 ± 0.1	103.5 ± 0.4
3	53.1 ± 4.8	54.7 ± 4.9
		10^{-6} M AKH
0	10.9 ± 0.3	10.4 ± 0.1
1	73.1 ± 9.8	73.2 ± 10.9
2	130.0 ± 7.1	108.4 ± 3.1*
3	62.1 ± 1.0	63.2 ± 0.2
4	55.9 ± 2.5	53.2 ± 5.5

[§]Age expressed in days and FAS activity expressed in μmol/min/mg protein. Means ± S.E. of 5 to 15 determinations; *, $P < 0.05$.

3.1.2.4. Triacylglycerol lipase

TGL from cricket fat body was significantly activated by *in vitro* treatment of the fat body with AKH, in the incubation medium MM199. When fat bodies from adult females were treated with AKH for 20, 40, 60 and 80 min, highest activation of TGL was attained after 40 min of treatment. AKH concentrations from 10^{-9} to 10^{-6} M were tested, and 10^{-7} M AKH was found to activate TGL the most. Age-dependent changes in the activity and sensitivity of TGL to 10^{-7} M AKH treatment of the fat body *in vitro* were studied in last larval instar (LL) and adult female crickets (Fig. 3). In LL, TGL activity showed a small peak on day 1 (3.10 nmol/h/mg protein) and then decreased gradually until day 7. Just before imaginal molt, TGL activity sharply increased. In adults, TGL activity was low on day 0, increased gradually until day 10 (6.67 nmol/h/mg protein) and then decreased slowly. The average basal TGL activity was much lower in the LL crickets compared to the adults. AKH significantly activated TGL in LL on day 2; in adult females the effect was significant from day 1.5 to 3. Typically, the fat bodies showed higher sensitivity to AKH during initial periods of both stages. AKH treatment of the fat body resulted in maximal activation of TGL by $29.8 \pm 10.7\%$ in the LL compared to $72.6 \pm 10.1\%$ in adults.
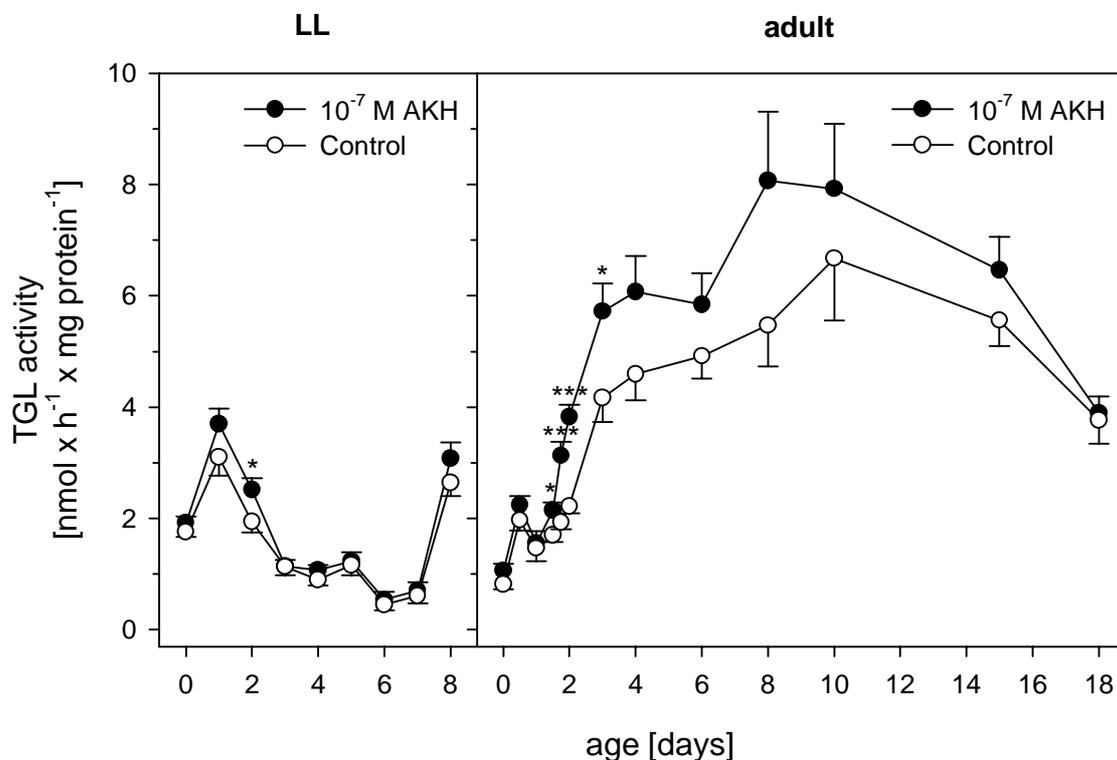

Fig. 3: Age-dependent effect of AKH treatment on the fat body TGL activity *in vitro*. Fat bodies from LL and adult female crickets were treated with 10^{-7} M AKH. Means $\pm$ S.E of 12 to 48 determinations; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Topical application of 100 pmol AKH on 3 d old adult female crickets led to a highly significant increase in the hemolymph lipid titer 2.5 h after the application. However, TGL extracted from the fat bodies dissected from the same insects showed no significant activation compared to the controls (Fig. 4).
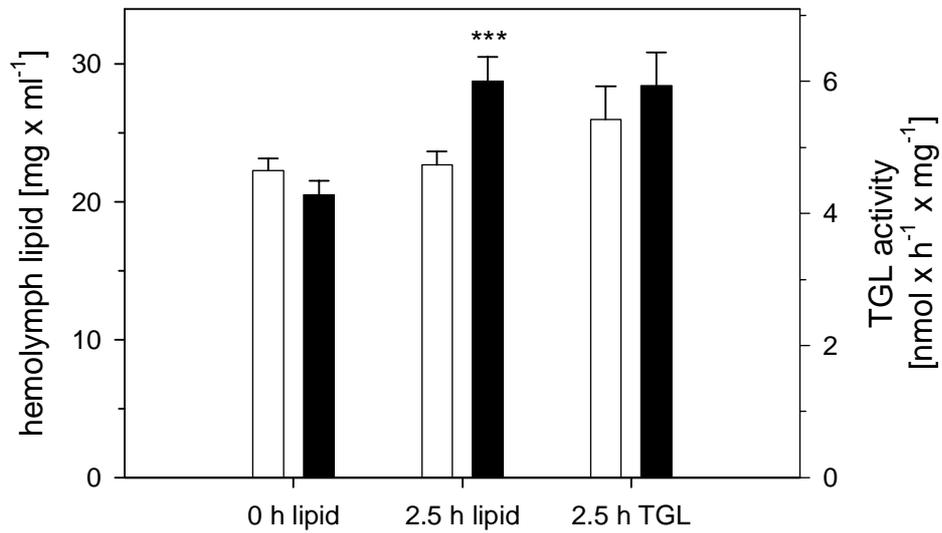

Fig. 4: Hemolymph lipid titer and TGL activity after topical application of 100 pmol AKH in 3 d old adult females. Lipid titer and TGL activity were determined for control (white bars) and AKH-treated insects (black bars). To determine the significance of difference, the titers were compared at 0 and 2.5 h for same insect. Means ± S.E. of 50 determinations; ***, $P < 0.001$.

3.2. FAS and acetate incorporation activity

Age-dependent changes in the FAS activity and acetate incorporation into lipid were studied in the adult female cricket fat body *in vitro* (Fig. 5). Both increased quickly and peaked soon after adult emergence. This was followed by sharp decline to basal level. The age-dependent patterns of acetate incorporation and of FAS activity showed high correlation (r = 0.7361, P = 0.00205).
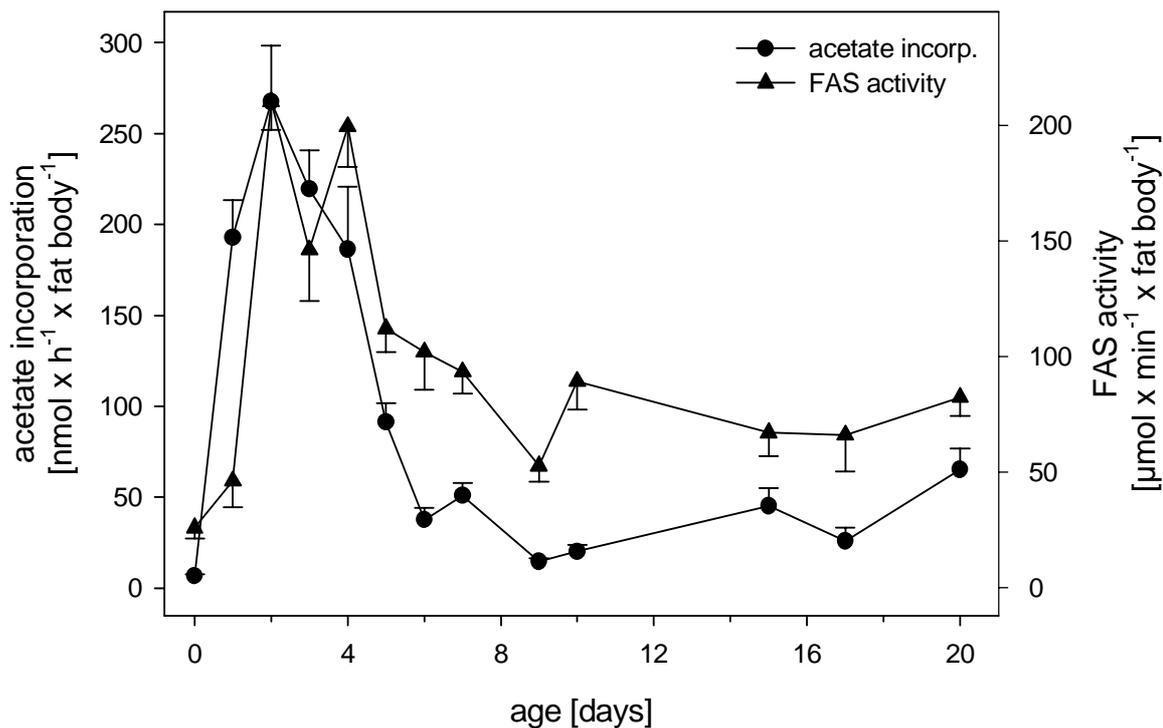

Fig. 5: Age-dependent acetate incorporation activity (circles) and FAS activity (triangles) *in vitro* for adult female cricket fat bodies. For acetate incorporation activity the incubations were carried out in MM199. Means ± S.E. of 10 to 30 determinations.

3.3. Signal transduction

3.3.1. Calcium

3.3.1.1. Role of Ca_e^{2+} in AKH effect on acetate incorporation

The role of Ca_e^{2+} in the inhibition of acetate incorporation by AKH was studied in adult female crickets. The fat bodies were treated with 10^{-8} M AKH in either FBIM+EGTA or FBIM. The inhibitory effect of AKH was abolished when the fat bodies were treated in the absence of Ca_e^{2+} (Fig. 6). Increasing the concentration of Ca_e^{2+} from 3 to 10 mM had no significant effect on the inhibition by AKH (data not shown).
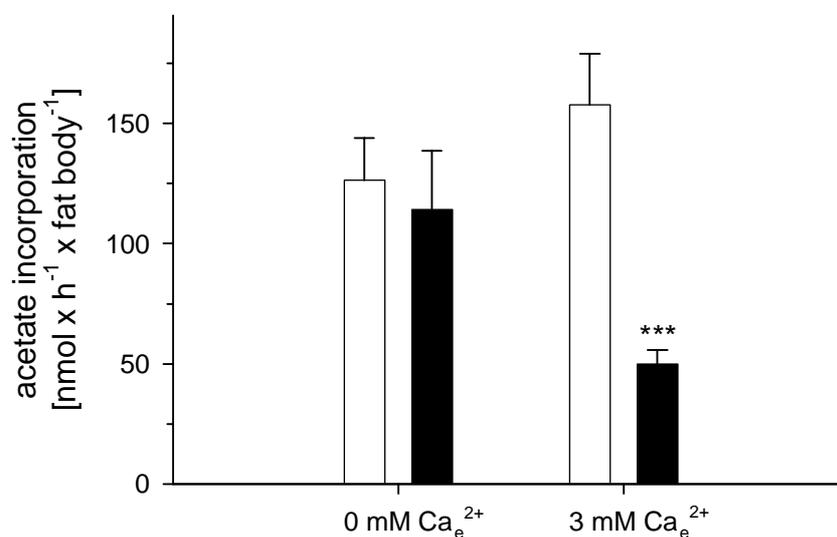

Fig. 6: Acetate incorporation in the adult female cricket fat body *in vitro* on incubation either with 10^{-8} M AKH (black bars) or without AKH (white bars) in FBIM+EGTA (0 mM Ca_e^{2+}) and FBIM (3 mM Ca_e^{2+}). FBIM+EGTA contained 1 mM EGTA and no Ca^{2+}. Means ± S.E. of 20 determinations; ***, $P < 0.001$.

3.3.1.2. Effects of Ca^{2+} chelators on acetate incorporation

The effects of Ca^{2+} chelators BAPTA, BAPTA-AM (a membrane-permeable form of BAPTA) and Quin 2-AM (also membrane-permeable) on the inhibitory effect of AKH on acetate incorporation were studied in adult female crickets. Ca^{2+} chelators showed no effect on the AKH action on fat bodies in FBIM (Table 8). Since at higher concentrations (0.5 mM), BAPTA and BAPTA-AM inhibited acetate incorporation substantially, they were tested at lower concentrations in combination with AKH.

Table 8:   Effects of Ca^{2+} chelators and AKH on acetate incorporation[§]

| Ca^{2+} chelator | Acetate incorporation [nmol/h/fat body] | | | |
	Control	Ca^{2+} chelator [mM]	AKH [M]	Ca^{2+} chelator [mM] + AKH [M]
		0.1 mM	10^{-10} M	0.1 mM + 10^{-10} M
BAPTA	146.1 ± 11.2	130.7 ± 10.9	56.2 ± 8.9***	37.3 ± 6.9***
		0.2 mM	10^{-10} M	0.2 mM + 10^{-10} M
BAPTA-AM	299.8 ± 35.5	298.1 ± 41.7	155.7 ± 13.0***	158.2 ± 17.0**
		0.05 mM	10^{-8} M	0.05 mM + 10^{-8} M
Quin 2-AM	175.5 ± 12.8	160.2 ± 13.2	48.5 ± 3.8***	43.1 ± 5.0***

[§]Effects of AKH and Ca^{2+} chelators on acetate incorporation in adult female cricket fat bodies *in vitro* in the incubation medium FBIM. Means ± S.E. of 20 to 50 determinations; **, $P < 0.01$; ***, $P < 0.001$.

3.3.1.3. Effects of Ca^{2+} channel blockers on acetate incorporation

The effects of Ca^{2+} channel blockers nifedipine, verapamil, $LaCl_3$ and ruthenium red, on the inhibitory effect of AKH on acetate incorporation were studied in adult female crickets. AKH inhibited lipid synthesis by the fat body at around the same level both in normal medium (FBIM) and in the presence of Ca^{2+} channel blockers (Table 9).

Table 9:   Effects of Ca^{2+} channel blockers and AKH on acetate incorporation[§]

Ca^{2+} channel blocker	Acetate incorporation [nmol/h/fat body]			
	Control	Ca^{2+} channel blocker [mM]	AKH [M]	Ca^{2+} channel blocker [mM] + AKH [M]
Nifedipine		0.1 mM	10^{-10} M	0.1 mM + 10^{-10} M
	135.5 ± 13.0	114.6 ± 8.9	34.3 ± 3.2***	30.4 ± 2.1***
Verapamil		0.2 mM	10^{-10} M	0.2 mM + 10^{-10} M
	181.4 ± 12.5	180.4 ± 12.7	35.6 ± 3.0***	35.4 ± 3.1***
LaCl$_3$		5 mM	10^{-8} M	5 mM + 10^{-8} M
	323.2 ± 49.5	184.0 ± 24.8*	85.4 ± 9.9***	115.8 ± 12.5***
Ruthenium red		0.05 mM	10^{-11} M	0.05 mM + 10^{-11} M
	92.1 ± 15.4	103.7 ± 16.8	74.2 ± 12.2	70.0 ± 9.8

[§]Effects of AKH and Ca^{2+} channel blockers on acetate incorporation in adult female cricket fat bodies *in vitro*, in the incubation medium FBIM. Means $\pm$ S.E. of 30 to 60 determinations; *, $P < 0.05$; ***, $P < 0.001$.

3.3.1.4. Effects of TMB-8 HCl, dantrolene and AKH on acetate incorporation

The effects of TMB-8 HCl (an intracellular calcium antagonist), dantrolene (a Ca^{2+} release inhibitor) and AKH were studied in adult female crickets, in the incubation medium FBIM. None of the chemicals studied influenced acetate incorporation or interfered with the action of AKH on lipid synthesis by the fat body *in vitro* (Table 10).

Table 10:   Effects of TMB-8 HCl, dantrolene and AKH on acetate incorporation[§]

Acetate incorporation [nmol/h/fat body]			
Control	100 µM TMB-8 HCL	10^{-11} M AKH	100 µM TMB-8 HCL + 10^{-11} M AKH
105.8 ± 6.5	129.5 ± 10.7	84.7 ± 10.1*	91.8 ± 12.8*
Control	10 µM dantrolene	10^{-10} M AKH	10 µM dantrolene + 10^{-10} M AKH
70.7 ± 6.1	69.7 ± 7.1	47.3 ± 7.6**	48.5 ± 6.6*

[§]Effects of TMB-8 HCl, dantrolene and AKH on acetate incorporation in adult female cricket fat bodies *in vitro*, in the incubation medium FBIM. Means $\pm$ S.E. of 40 to 60 determinations; *, $P < 0.05$; **, $P < 0.01$.

3.3.1.5. Effect of thapsigargin on acetate incorporation

Thapsigargin releases Ca^{2+} from intracellular stores by irreversibly inhibiting sarco-endoplasmic reticulum Ca^{2+}-ATPases. The effect of thapsigargin on lipogenesis by fat bodies was studied in adult female crickets. Thapsigargin significantly inhibited acetate incorporation by the fat bodies only in the presence of Ca_e^{2+} (Fig. 7). The effect of thapsigargin was concentration dependent ($70.4 \pm 4.0\%$ inhibition at 0.2 mM thapsigargin).
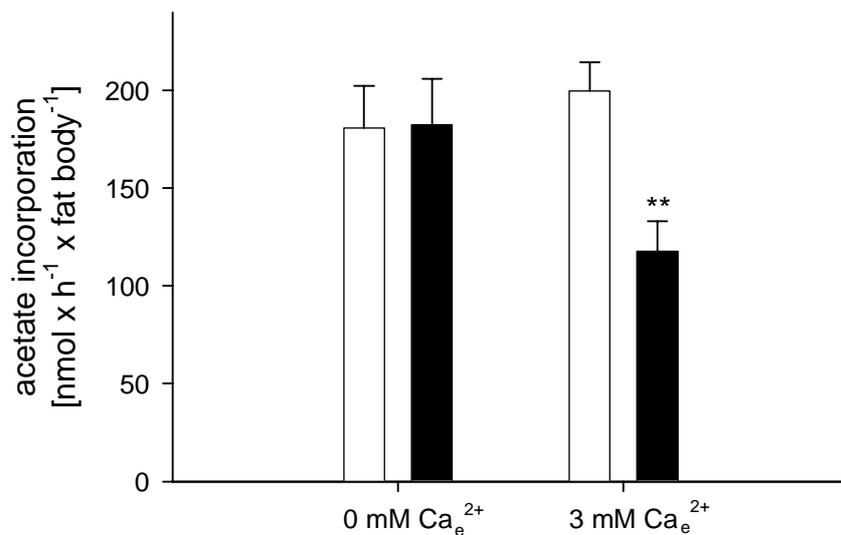

Fig. 7: Acetate incorporation in adult female cricket fat bodies *in vitro* on incubation with 10 μM thapsigargin (black bars) and without thapsigargin (white bars) in FBIM+EGTA (0 mM Ca_e^{2+}) and FBIM (3 mM Ca_e^{2+}). Means $\pm$ S.E. of 30 determinations; **, $P < 0.01$.

3.3.1.5.1. Reversibility of thapsigargin effect

Thapsigargin strongly inhibited acetate incorporation by fat bodies from adult female crickets *in vitro*. The fat bodies incubated with 50 µM thapsigargin failed to recover after incubation without thapsigargin for 60 min (Fig. 8) indicating that the inhibitory effect was irreversible.
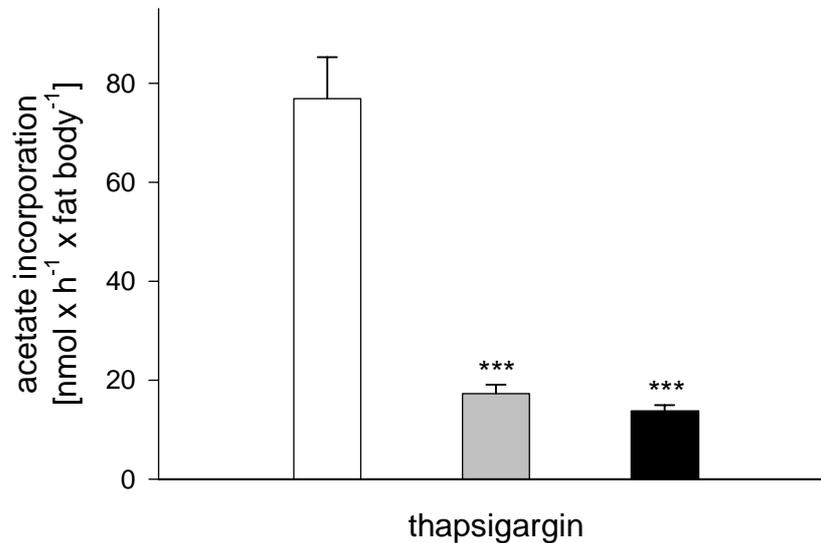

Fig. 8: Reversibility of acetate incorporation inhibition by thapsigargin, determined by incubating fat bodies in FBIM. One test and two control sets were incubated in the experiment. The test fat bodies (gray bar) were incubated as follows. Incubation 1: with 50 µM thapsigargin for 1 h. Incubation 2: without thapsigargin for 1 h. Incubation 3: without thapsigargin in [1-^{14}C]Na-acetate labeled medium for 30 min. Two control sets were incubated along with. For the first control set (white bar), the fat bodies were incubated three times as described for the test fat bodies, but without thapsigargin throughout the experiment. For the second control set (black bar), 50 µM thapsigargin was present during all three incubations. Means ± S.E. of 20 determinations; ***, $P < 0.001$.

3.3.1.5.2. Effects of thapsigargin, TMB-8 HCl and AKH

The effects of thapsigargin in combination with either AKH or with the intracellular calcium antagonist TMB-8 HCl on acetate incorporation were studied in adult female crickets (Table 11). The effects of thapsigargin and AKH were only additive and no synergism was observed. TMB-8 HCl showed no interference with the inhibition of acetate incorporation by thapsigargin.

Table 11:   Effects of thapsigargin, AKH and TMB-8 HCl on acetate incorporation[§]

Acetate incorporation [nmol/h/fat body]			
Control	2 µM thapsigargin	10^{-11} M AKH	2 µM thapsigargin + 10^{-11} M AKH
199.8 ± 14.8	153.5 ± 25.9**	125.9 ± 14.8***	76.5 ± 8.7***
Control	4 µM thapsigargin	0.1 mM TMB-8 HCl	4 µM thapsigargin + 0.1 mM TMB-8 HCl
332.2 ± 31.4	190.8 ± 22.3***	356.8 ± 39.5	189.4 ± 27.8**

[§]Effects of thapsigargin, AKH and TMB-8 HCl on acetate incorporation in adult female cricket fat bodies *in vitro*, in the incubation medium FBIM. Means ± S.E. of 20 to 60 determinations; **, P < 0.01; ***, P < 0.001.

3.3.1.6. Effects of Ca^{2+} ionophores on acetate incorporation

3.3.1.6.1. Effect of A23187

The effect of the calcium ionophore A23187 on acetate incorporation at various Ca_e^{2+} concentrations was studied in fat bodies from adult female crickets in the incubation media FBIM+EGTA and FBIM (Table 12). At 0.01 and 0.1 mM, A23187 inhibited acetate incorporation when Ca_e^{2+} concentrations in FBIM were 3 mM or higher.

Table 12:   Effect of A23187 on acetate incorporation[§]

Ca_e^{2+} [mM]	Acetate incorporation [nmol/h/fat body]			
	Control	A23187 [mM]		
		0.01	0.1	0.2
0	114.0 ± 11.3	–	110.1 ± 10.8	–
3	220.0 ± 16.1	155.6 ± 13.0*	115.7 ± 16.6***	188.2 ± 16.2
10	114.1 ± 16.2	–	55.3 ± 6.9**	–

[§]Effect of calcium ionophore, A23187 on acetate incorporation in adult female cricket fat bodies *in vitro*. Means ± S.E. of 30 to 60 determinations; *, P < 0.05; **, P < 0.01; ***, P < 0.001.

3.3.1.6.2. Effect of ionomycin

The effect of the calcium ionophore ionomycin on lipogenesis by fat bodies was studied in adult female crickets. Ionomycin significantly inhibited acetate incorporation by the fat bodies only in the presence of Ca_e^{2+} (Fig. 9). The effect of ionomycin was concentration dependent ($73.5 \pm 3.1\%$ inhibition at 100 µM ionomycin).
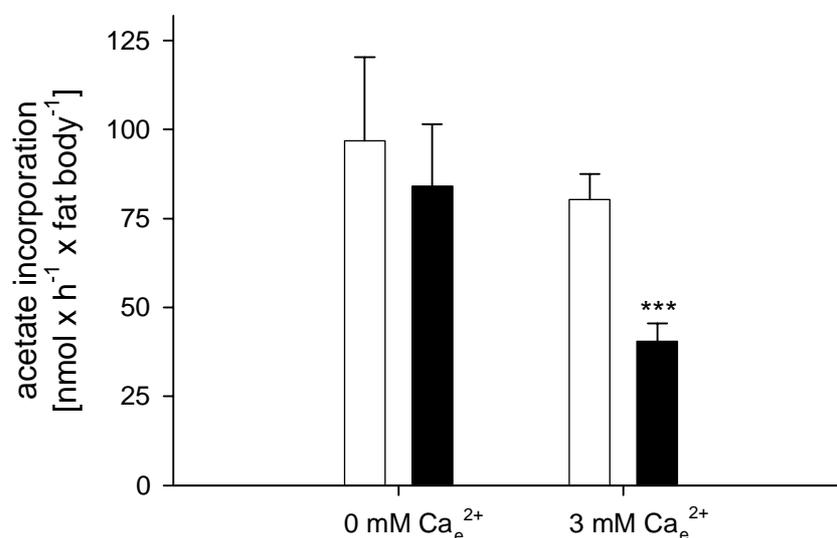

Fig. 9: Acetate incorporation in the adult female cricket fat body *in vitro* on incubation with 10 µM ionomycin (black bars) and without ionomycin (white bars) in FBIM+EGTA (0 mM Ca_e^{2+}) and FBIM (3 mM Ca_e^{2+}). Means $\pm$ S.E. of 20 to 40 determinations; ***, P < 0.001.

3.3.1.6.2.1. Effects of ionomycin, TMB-8 HCl and AKH

The effect of ionomycin in combination either with AKH or with TMB-8 HCl on acetate incorporation was studied in fat bodies from adult female crickets in the incubation medium FBIM (Table 13). No synergistic effect of ionomycin and AKH was observed. TMB-8 HCl showed no interference in the inhibition of acetate incorporation by ionomycin.

Table 13:   Effects of ionomycin, AKH and TMB-8 HCl on acetate incorporation[§]

Acetate incorporation [nmol/h/fat body]			
Control	2 µM ionomycin	10^{-11} M AKH	2 µM ionomycin + 10^{-11} M AKH
80.4 ± 7.1	61.3 ± 8.8	$45.1 \pm 6.7*$	$42.2 \pm 7.2*$
Control	5 µM ionomycin	0.1 mM TMB-8 HCl	5 µM ionomycin + 0.1 mM TMB-8 HCl
84.4 ± 8.9	$54.0 \pm 6.1*$	73.2 ± 8.5	$54.8 \pm 6.6**$

[§]Effects of ionomycin, AKH and TMB-8 HCl on acetate incorporation in adult female cricket fat bodies *in vitro*. Means $\pm$ S.E. of 20 to 60 determinations; *, P < 0.05; **, P < 0.01.

3.3.1.6.2.2. Reversibility of ionomycin effect

Ionomycin strongly inhibited acetate incorporation by the adult female cricket fat body *in vitro*. The fat bodies incubated with 50 µM ionomycin showed complete recovery after incubation without ionomycin for 60 min (Fig. 10), indicating that the inhibitory effect was reversible.
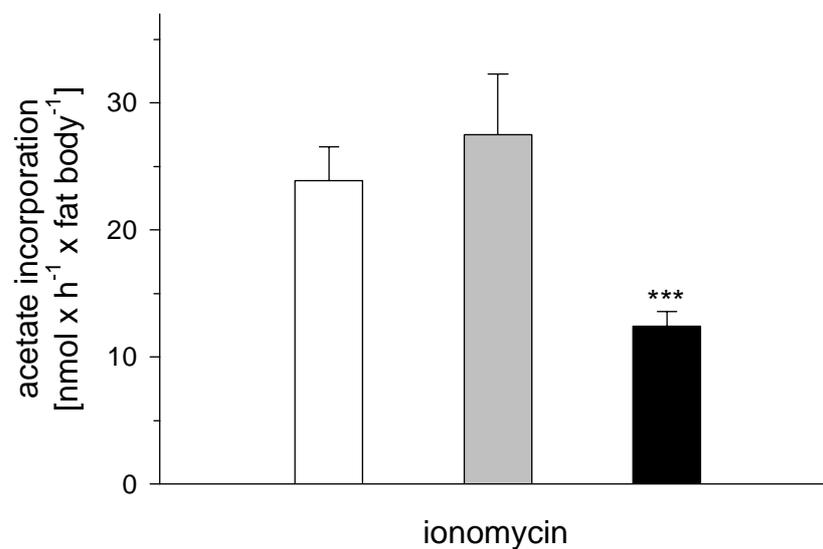

Fig. 10: The reversibility of acetate incorporation inhibition by ionomycin, determined by incubating fat bodies in FBIM. One test and two control sets were incubated in the experiment. The test fat bodies (gray bar) were incubated as follows. Incubation 1: with 50 µM ionomycin for 1 h. Incubation 2: without ionomycin for 1 h. Incubation 3: without ionomycin in [1-^{14}C]Na-acetate labeled medium for 30 min. Two control sets were incubated along with. For the first control set (white bar), the fat bodies were incubated three times as described for the test fat bodies, but without ionomycin throughout the experiment. For the second control set (black bar), 50 µM ionomycin was present during all three incubations. Means ± S.E. of 40 determinations; ***, $P < 0.001$.

3.3.1.7. Effects of suramin and AKH on acetate incorporation

Suramin, a non-selective P2 purinergic antagonist, is known to activate Ca^{2+} release from skeletal muscle sarcoplasmic reticulum by activating ryanodine receptors. The effects of suramin and AKH on acetate incorporation by fat bodies from adult female crickets were studied. Suramin alone had no effect on lipid synthesis but antagonized the AKH effect to a small extent (Fig. 11).
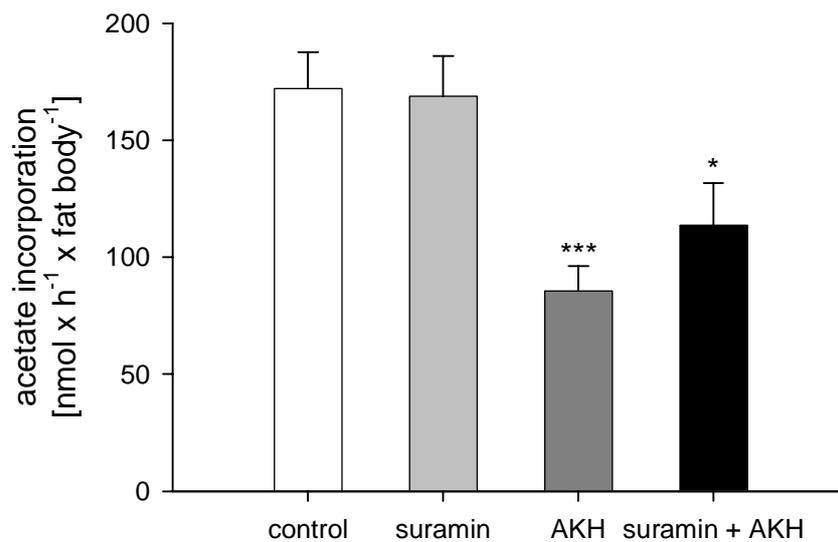

Fig. 11: Effects of suramin and AKH on acetate incorporation in adult female crickets. Fat bodies were treated either with 50 µM suramin (gray bar) or 10^{-11} M AKH (dark gray bar) or both (black bar) in FBIM. Means ± S.E. of 20 determinations; *, $P < 0.05$; ***, $P < 0.001$.

3.3.1.8. Influx and efflux studies

3.3.1.8.1. Effect of AKH on influx of acetate and Ca^{2+}

Fat bodies from adult female crickets were treated with AKH in [1-^{14}C]Na-acetate- or ^{45}CaCl$_2$-labeled MM199, to study the effect of AKH on the influx of acetate and Ca^{2+} respectively. No significant effect of AKH was observed on the influx of either at the concentrations and incubation periods studied (Table 14).

Table 14:   Effect of AKH on influx of acetate and Ca^{2+} into fat body[§]

Chemical	Incubation period [min]	Radioactivity in aqueous phase [dpm/fat body]	
		Control	Test
			10^{-7} M AKH
Acetate	2	1218.0 ± 193.0	1375.2 ± 301.5
Ca^{2+}	1	19026.4 ± 1779.2	14833.6 ± 1119.2
			10^{-8} M AKH
Ca^{2+}	0.33	11193.6 ± 1360.0	9283.2 ± 1136.0
	1	17139.2 ± 1299.2	14536.8 ± 1030.4
	4	31869.6 ± 2828.8	28070.4 ± 2433.6

[§]Effect of AKH on the influx of acetate and Ca^{2+} in adult female cricket fat bodies *in vitro*. The radioactivity recovered in the aqueous phase after extraction of the fat bodies is presented in dpm/fat body. Means $\pm$ S.E. of 10 to 20 determinations.

3.3.1.8.2. Effect of AKH on efflux of Ca^{2+}

The effect of AKH on the efflux of Ca^{2+} from the adult female cricket fat body was studied. Fat bodies were labeled with ^{45}CaCl$_2$ by incubating in 100 µl MM199 containing ^{45}CaCl$_2$. The fat bodies were then treated with AKH in non-radiolabeled MM199 to study its effect on the efflux of Ca^{2+}. The comparison of ^{45}Ca^{2+} released during Efflux1 (without AKH) and Efflux2 (with AKH) was used to determine the effect of AKH on ^{45}Ca^{2+} efflux from the fat body. For the control fat bodies, both Efflux1 and Efflux2 were studied by incubation in medium without AKH. No significant effect of AKH was observed on the efflux of Ca^{2+} at the concentrations and incubation periods studied (Table 15).

Table 15:   Effect of AKH on efflux of Ca^{2+} from fat body[§]

| Incubation period [min] | Radioactivity released [dpm/100 µl MM199] | | | |
| | Control | | Test | |
	Efflux1	Efflux2	Efflux1	Efflux2
				10^{-7} M AKH
2	3299.0 ± 435.0	3180.6 ± 397.2	3185.9 ± 504.9	3660.0 ± 260.0
				10^{-8} M AKH
0.5	1295.7 ± 203.7	1455.4 ± 132.2	1852.6 ± 537.4	1198.6 ± 202.3
2	1920.7 ± 320.0	1905.8 ± 325.5	1830.6 ± 357.4	1919.0 ± 350.6
4	3703.3 ± 540.2	4113.8 ± 833.6	3207.6 ± 382.0	3304.8 ± 553.0

[§]Effect of AKH on efflux of Ca^{2+} from adult female cricket fat bodies *in vitro*. The radioactivity released in the medium is presented as dpm/100 µl. Means $\pm$ S.E. of 8 determinations.

3.3.1.9. Effects of caffeine, theophylline, thapsigargin and ionomycin on TGL activity

The effects of caffeine, theophylline, thapsigargin and ionomycin were studied on the fat body TGL from adult female crickets. At 3 and 10 mM Ca_e^{2+} concentrations, caffeine, theophylline and thapsigargin showed a tendency towards activation of TGL (Fig. 12). However, the effect was significant only in the case of theophylline at 3 mM Ca_e^{2+}. Ionomycin treatment showed a tendency towards inhibition of TGL at 3 mM Ca_e^{2+} and activation of TGL at 10 mM Ca_e^{2+}, however neither was statistically significant.

Significant activation of TGL (by $42.3 \pm 13.5\%$; $P < 0.01$) was observed when the fat bodies were treated with 50 mM caffeine at 3 mM Ca_e^{2+}. The activation was not significant when the fat bodies were treated with 30 mM theophylline (by $23.1 \pm 12.3\%$), 50 µM thapsigargin (by $61.1 \pm 21.7\%$) or 50 µM ionomycin (by $9.7 \pm 11.2\%$), nevertheless there was a tendency towards activation.
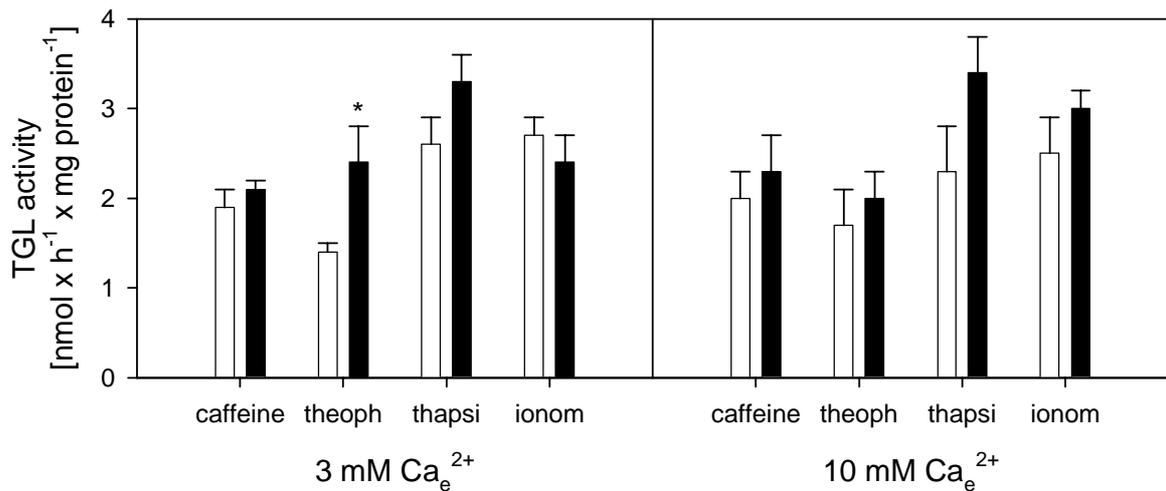
Fig. 12: TGL activity on incubation of the fat body with either caffeine, theophylline, thapsigargin or ionomycin. The fat bodies from adult female crickets were incubated with 10 mM caffeine, 10 mM theophylline, 4 μM thapsigargin or 5 μM ionomycin (black bars) at 3 and 10 mM Ca_e^{2+} in FBIM. Controls are presented by white bars. Means ± S.E. of 12 determinations; *, $P < 0.05$.

3.3.2. cAMP

3.3.2.1. Effects of caffeine and theophylline on acetate incorporation

Caffeine and theophylline increase cellular cAMP levels by inhibiting the phosphodiesterase. Caffeine is also known to affect cellular calcium levels through the release of calcium from intracellular stores. The effects of caffeine and theophylline on acetate incorporation were studied in adult female cricket fat bodies *in vitro*. Highly significant inhibition of acetate incorporation was observed upon caffeine or theophylline treatment of the fat body in the incubation medium FBIM (Fig. 13).
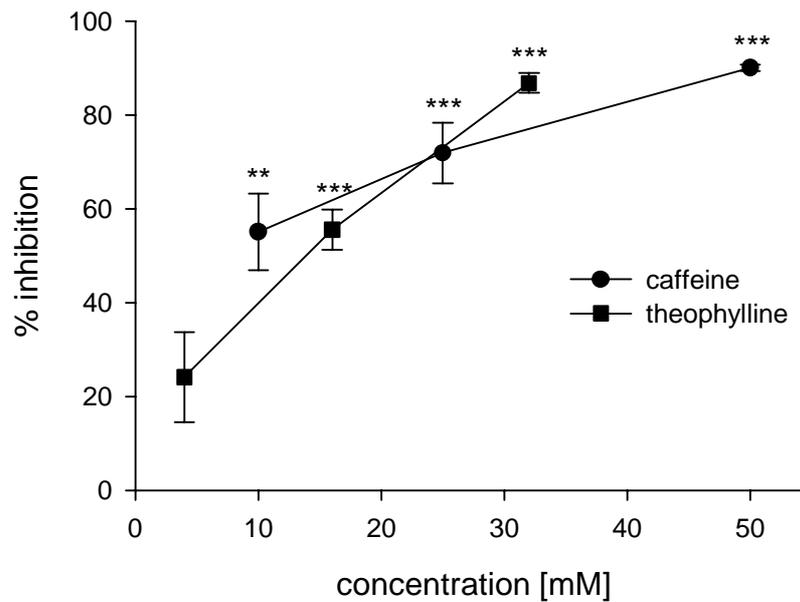
Fig. 13: Inhibition of acetate incorporation by caffeine in adult female cricket fat bodies *in vitro*. Control fat bodies incorporated 211.0 ± 24.1 nmol acetate/h/fat body on incubation in FBIM without caffeine and theophylline. Means $\pm$ S.E. of 10 to 20 determinations; **, $P < 0.01$; ***, $P < 0.001$.

3.3.2.1.1. Role of Ca_e^{2+} in caffeine and theophylline effects

Fat bodies from adult female crickets were treated with caffeine or theophylline in FBIM with and without Ca_e^{2+}, to study its possible role in their effects on the fat body. Inhibition of acetate incorporation by caffeine or theophylline was not affected by the removal of Ca_e^{2+} (Fig. 14).

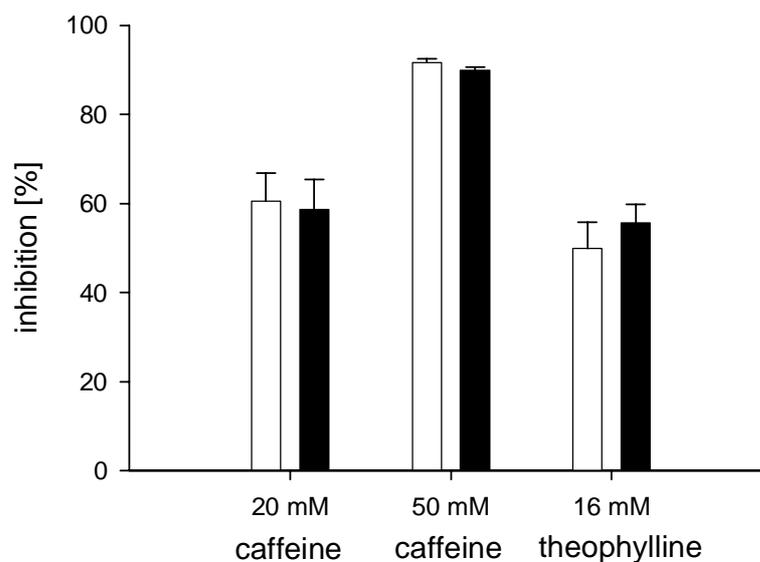Fig. 14: Inhibition of acetate incorporation in adult female cricket fat bodies *in vitro* upon incubation with 20 and 50 mM caffeine or 16 mM theophylline in FBIM with Ca_e^{2+} (black bars) and without Ca_e^{2+} (white bars). Control fat bodies incubated in FBIM with and without Ca_e^{2+} incorporated 211.0 ± 24.1 and 219.9 ± 80.7 nmol acetate/h/fat body, respectively. Means $\pm$ S.E. of 10 to 20 determinations.

3.3.2.1.2. Reversibility of caffeine and theophylline effects

Caffeine and theophylline strongly inhibited acetate incorporation by the cricket fat body *in vitro*. To check whether this effect was nonspecific/toxic, the ability of fat bodies to recover after incubation with high concentrations of caffeine/theophylline was tested. Fat bodies incubated with either 50 mM caffeine or 32 mM theophylline showed good recovery after incubation without caffeine/theophylline for 60 min (Fig. 15), indicating that the inhibitory effect was reversible.
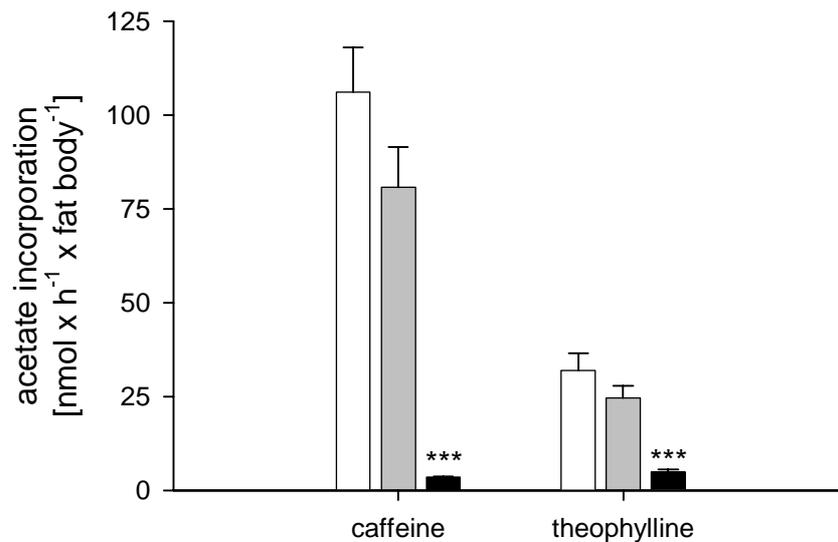
Fig. 15: Reversibility of acetate incorporation inhibition by caffeine and theophylline, determined by incubating fat bodies in FBIM. One test and two control sets were incubated in the experiment. The test fat bodies (gray bars) were incubated as follows. Incubation 1: with 50 mM caffeine or with 32 mM theophylline for 1 h. Incubation 2: without caffeine/theophylline for 1 h. Incubation 3: without caffeine/theophylline in [1-^{14}C]Na-acetate labeled medium for 30 min. Two control sets were incubated along with. For the first control set (white bars), the fat bodies were incubated three times as described for the test fat bodies, but without caffeine/theophylline throughout the experiment. For the second control set (black bars), 50 mM caffeine or 32 mM theophylline was present during all three incubations. Means ± S.E. of 40 determinations; ***, P < 0.001.

3.3.2.1.3. Effects of caffeine and AKH

The effects of caffeine and AKH on acetate incorporation by the fat body were studied independently and in combination with each other. At low concentrations, when caffeine and AKH were used independently, both inhibited acetate incorporation by the fat body to a small extent. However, they showed only an additive but no synergistic effect when used in combination (Fig. 16).
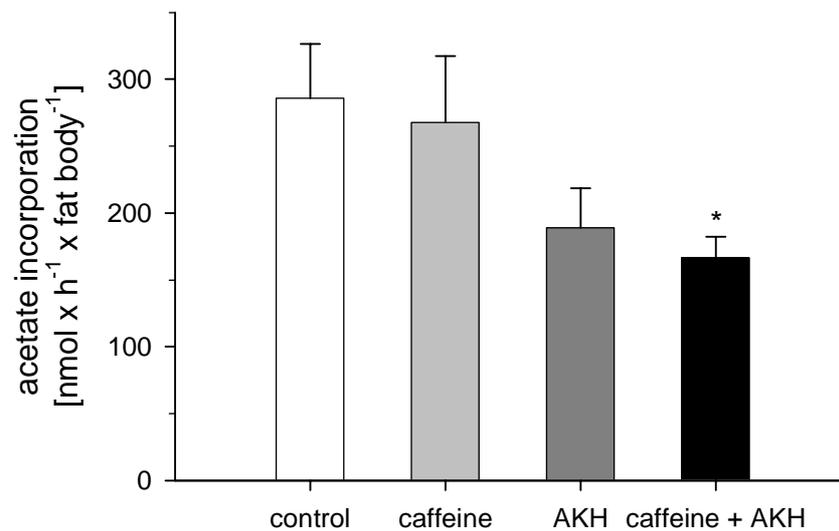
Fig. 16: Effects of caffeine and AKH on acetate incorporation in adult female crickets. Fat bodies were treated either with 5 mM caffeine (gray bar) or 10^{-11} M AKH (dark gray bar) or both (black bar) in FBIM. Means ± S.E. of 10 determinations; *, $P < 0.05$.

3.3.2.1.4. Effects of ryanodine, caffeine and AKH

The effect of Ca^{2+} release inhibitor, ryanodine on acetate incorporation was studied in adult female crickets. The fat bodies from adult females were treated with ryanodine and caffeine in FBIM, independently and in combination with each other. The fat bodies were also treated independently and in combination with each other, with ryanodine and AKH. Ryanodine did not interfere with the action of caffeine, but lowered the inhibition of lipid synthesis by AKH to some extent (Fig. 17). Ryanodine, at a concentration range of 10^{-10} to 2×10^{-5} M , had no significant effect on fat bodies when used independently (data not shown).
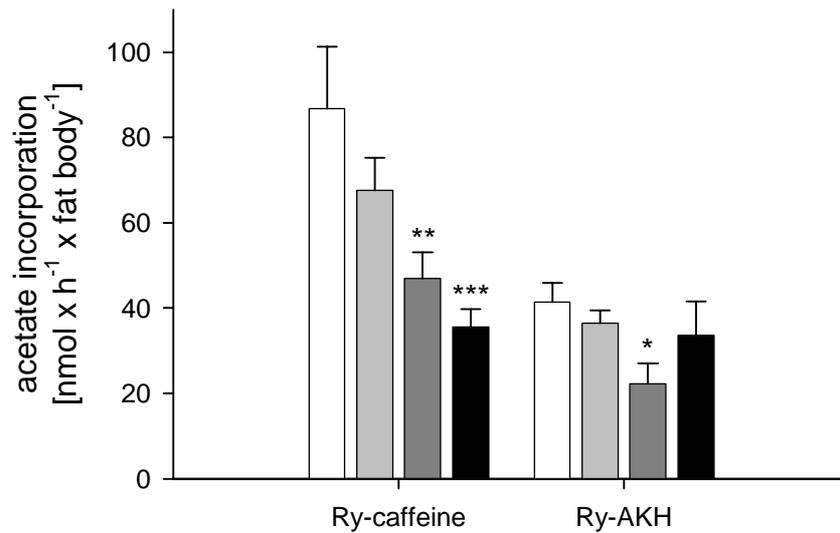
Fig. 17: Effects of ryanodine, caffeine and AKH on acetate incorporation in adult female crickets. Ry-caffeine: The fat bodies were treated either with 2×10^{-5} M ryanodine (gray bar) or 20 mM caffeine (dark gray bar) or both (black bar) in FBIM. Ry-AKH: The fat bodies were treated either with 2×10^{-5} M ryanodine (gray bar) or 10^{-9} M AKH (dark gray bar) or both (black bar) in FBIM. The controls are presented by white bars. Means ± S.E. of 40 to 60 determinations; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

3.3.2.2. Effects of cAMP analogues on acetate incorporation

The effects of various cell-permeable cAMP analogues together with 1 mM IBMX (a phosphodiesterase inhibitor) on acetate incorporation were studied in adult female crickets. None of the analogues studied showed significant effect on fat bodies from 1 d old females, in the incubation medium FBIM *in vitro* (Fig. 18). No consistent effect of 1 mM IBMX with 0.2 mM 8-Br-cAMP was observed on fat bodies from 6 and 12 h old adult females (data not shown). The effects were not significant when Sp-5,6-DCI-cBIMPS and db-cAMP were tested independently on fat bodies from adult females in the concentration ranges 0.02 to 0.5 mM and 0.01 to 0.2 mM respectively (data not shown).
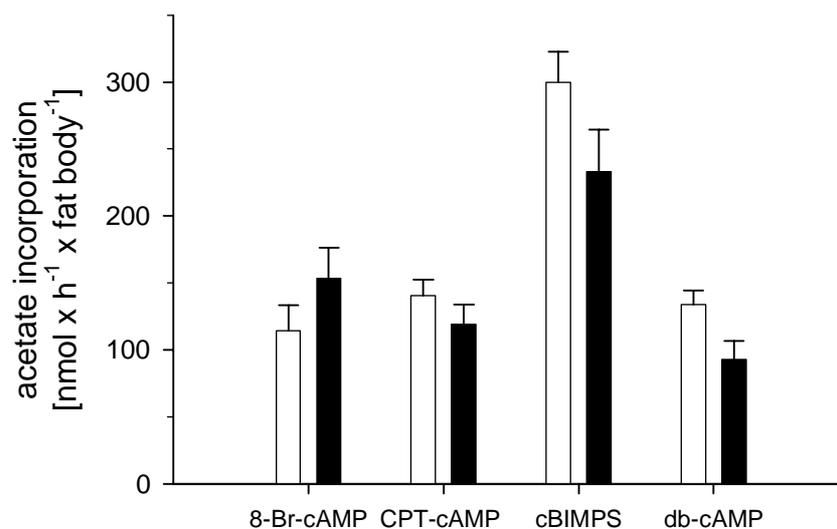
Fig. 18: Acetate incorporation in the adult female cricket fat body *in vitro* on incubation with 1 mM IBMX and 0.2 mM 8-Br-cAMP (8-Br-cAMP, black bar), 0.1 mM 8-CPT-cAMP (CPT-cAMP, black bar), 0.2 mM Sp-5,6-DCI-cBIMPS (cBIMPS, black bar) or 0.2 mM db-cAMP (db-cAMP, black bar), respectively. Controls are presented by white bars. Means ± S.E. of 40 to 60 determinations.

3.3.2.3. Effects of forskolin, cholera toxin, IBMX and AKH on acetate incorporation

The effects of forskolin, cholera toxin and IBMX on acetate incorporation by the fat body in adult females were studied in FBIM. Forskolin and cholera toxin activate adenylate cyclase, while IBMX inhibits phosphodiesterase, and thereby increase intracellular cAMP levels. Cholera toxin significantly inhibited lipid synthesis, forskolin and IBMX showed a non-significant tendency towards inhibition (Table 16).

Table 16:   Effects of forskolin, cholera toxin and IBMX on acetate incorporation[§]

Acetate incorporation [nmol/h/fat body]			
Control	0.1 mM forskolin	1 mM IBMX	0.1 mM forskolin + 1 mM IBMX
221.6 ± 31.6	196.8 ± 29.3	168.6 ± 18.4	179.3 ± 24.3
Control	50 µg/ml cholera toxin	1 mM IBMX	50 µg/ml cholera toxin + 1 mM IBMX
400.4 ± 28.2	297.0 ± 19.8**	373.7 ± 28.6	306.3 ± 23.5*
Control	10^{-11} M AKH	1 mM IBMX	10^{-11} M AKH + 1 mM IBMX
270.4 ± 24.2	201.6 ± 23.9	231.8 ± 28.1	225.7 ± 22.9

[§]Effects of forskolin, cholera toxin and IBMX on acetate incorporation in adult female cricket fat bodies *in vitro*. Means ± S.E. of 20 to 40 determinations; *, $P < 0.05$; **, $P < 0.01$.

3.3.2.4. Effects of AKH, forskolin and IBMX on intracellular cAMP levels

The effects of AKH, forskolin and IBMX on intracellular cAMP levels were studied in adult female crickets. There was almost no change in the cAMP levels after treatment of the fat bodies with 10^{-7} M AKH in FBIM *in vitro* (Fig. 19). However, a highly significant increase was observed upon 15 min incubation of the fat bodies with 1 mM IBMX (value at 0 min for 1 mM IBMX + 0.1 mM forskolin in Fig. 19). The fat bodies incubated with IBMX were transferred to the medium containing 1 mM IBMX and 0.1 mM forskolin, which resulted into further increase in the cAMP levels with time. The time point, at which the fat bodies were transferred to the medium containing IBMX and forskolin, corresponds to the point 0 min in the fig. 19.
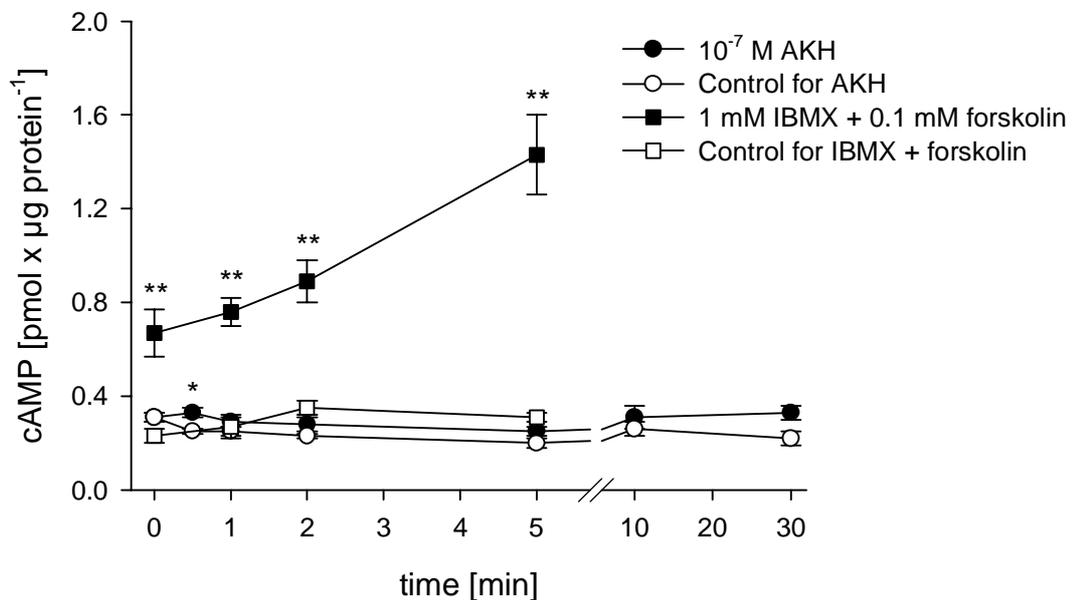
Fig. 19: The effects of AKH, forskolin and IBMX on intracellular cAMP levels in the fat bodies of adult female crickets *in vitro*. Means ± S.E. of 8 (for AKH treatment) and 6 (for IBMX and forskolin treatment) determinations; *, $P < 0.05$; **, $P < 0.01$.

3.3.3. Diacylglycerol

3.3.3.1. Effects of PMA and AKH on acetate incorporation

PMA (a phorbol ester) activates protein kinase C by virtue of its resemblance with diacylglycerol. The effects of PMA and AKH on acetate incorporation by fat bodies from adult female crickets were studied. PMA alone had no effect on lipid synthesis and also in combination with AKH (Fig. 20).
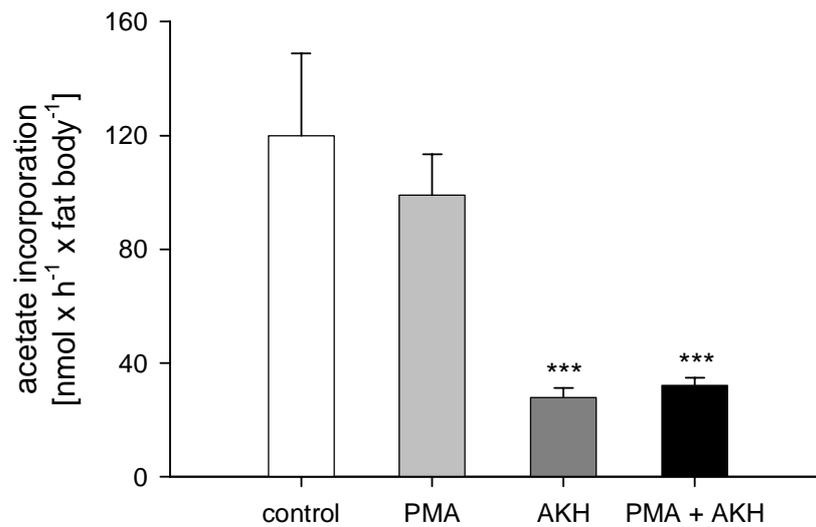
Fig. 20: Effects of PMA and AKH on acetate incorporation in adult female crickets. Fat bodies were treated either with 10 μM PMA (gray bar) or 10^{-10} M AKH (dark gray bar) or both (black bar) in FBIM. Means ± S.E. of 20 determinations; ***, $P < 0.001$.

3.4. Penultimate larval instar crickets

3.4.1. Effect of AKH on acetate incorporation

Age-dependent activity of acetate incorporation into lipid by the fat body from PL male and female crickets was studied using acetate as a radiolabeled precursor. Inhibition of acetate incorporation by 10^{-7} M AKH was also studied (Fig. 21). Acetate incorporation activity increased sharply and peaked on day 2. It was significantly inhibited by AKH throughout almost the entire duration of the instar, in both male and female crickets.
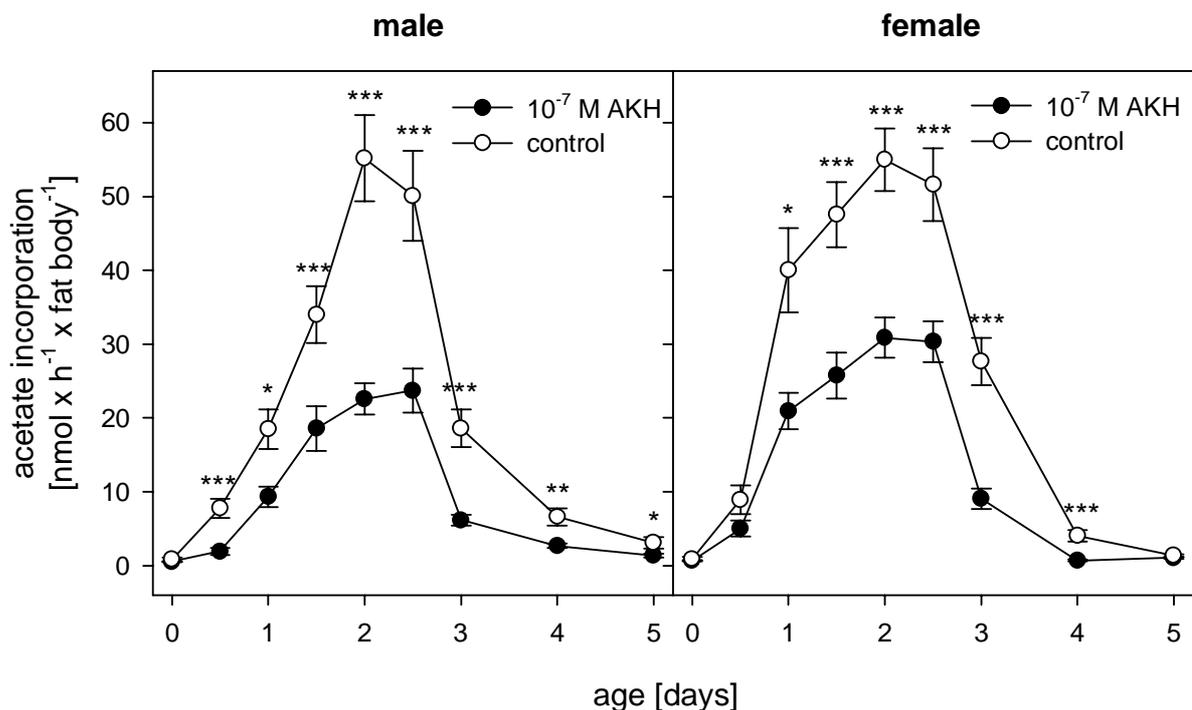
Fig. 21: Age-dependent effect of AKH treatment on fat body lipogenic activity *in vitro*. Fat bodies from PL male and female crickets were treated with 10^{-7} M AKH in the incubation medium FBIM. Means $\pm$ S.E of 40 to 60 determinations; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

3.4.1.1. Dose-response for PL females

Concentration dependent inhibition of acetate incorporation by AKH was studied in PL female cricket fat bodies. A sigmoidal dose-response curve for inhibition was obtained when AKH was used in a concentration range of 10^{-15} to 10^{-4} M (Fig. 22). The EC_{50} for the inhibitory effect was 7.9×10^{-11} M AKH, while the maximum inhibition of $83.4 \pm 1.8\%$ was reached at 10^{-4} M. Acetate incorporation was significantly inhibited by AKH at concentrations as low as 10^{-14} M.
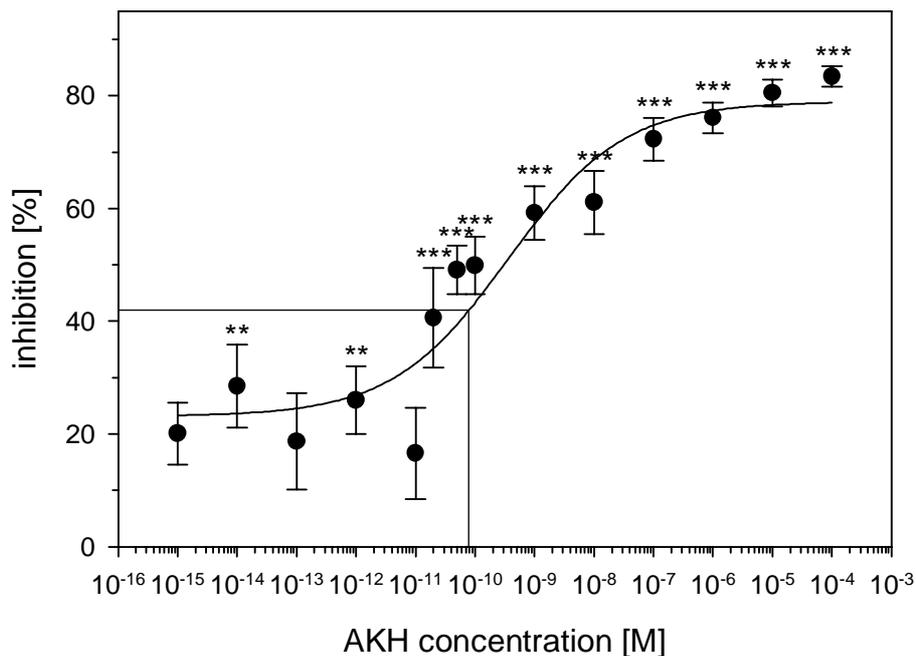
Fig. 22: Dose-response for the inhibition of acetate incorporation into lipid by 2 d old PL female cricket fat bodies treated with AKH in the incubation medium MM199 *in vitro*. Control fat bodies incorporated 158.82 ± 7.0 nmol acetate/h/fat body. Means ± S.E of 20 to 40 determinations; *, P < 0.05; **, P < 0.01; ***, P < 0.001. Non-linear regression was performed with the Hill equation ($y = y_0 + ax^b/(c^b + x^b)$); a = 55.79, b = 0.45, c = 0 and y_0 = 23.14), using SigmaPlot 7.0. The r² of the regression curve was 0.923.

3.4.2. Fat body composition

Age-dependent changes in the fat body composition of PL female crickets were as follows. Lipid and protein content of the fat body significantly increased from day 0 to day 3 (Fig. 23a, for the statistical analysis see Table 18). After peaking on day 3, lipid content remained at more or less the same level for the rest of the instar, but protein content decreased significantly. Glycogen content of the fat body increased significantly to a peak on day 4 and then declined significantly on the fifth day. Free carbohydrate content and fat body wet weight showed a significant increase throughout the larval stage and reached maximum levels on day 5 (Fig. 23b and c, Table 17). Lipid and protein together formed almost 46% of the wet weight of the fat body on day 3 (Table 17). In general, lipid and protein were the principal contributors, while glycogen and free carbohydrate together contributed less than 10% of the fat body wet weight at any time point of the larval instar (Table 17).

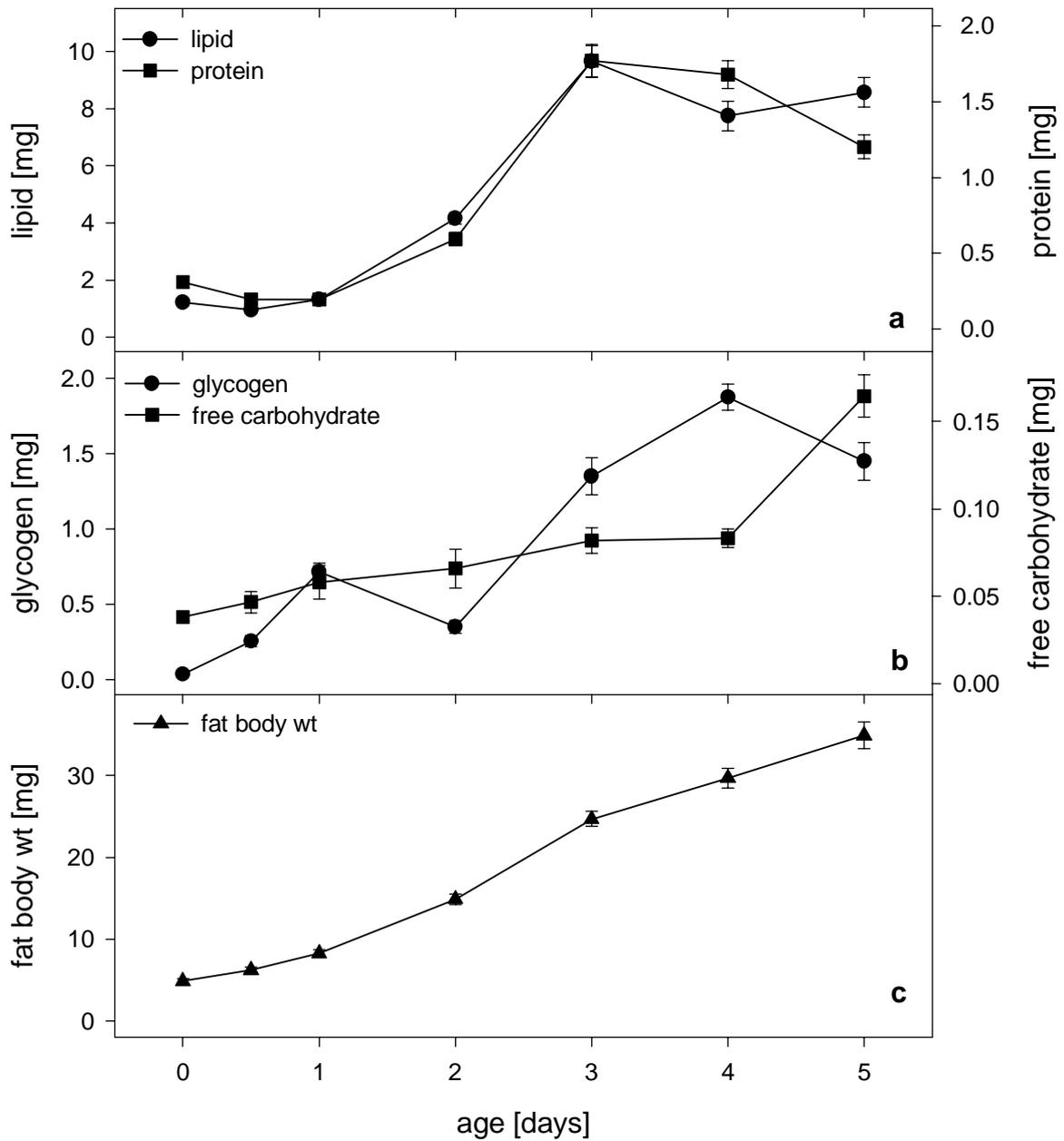Fig. 23: Age-dependent composition and weight of fat bodies from PL female crickets. All values are presented on a per animal basis. **a:** Total amount of lipid (circles) and protein (squares) in mg. **b:** Total amount of glycogen (circles) and free carbohydrates (squares) in mg. **c:** Fat body weight in mg (triangles). Means ± S.E. of 20 to 30 determinations.

Table 17:   Age-dependent changes in fat body composition[§]

Age	Lipid	Protein	Glycogen	Free carbohydrate	Water[#]
[d]	[%]	[%]	[%]	[%]	[%]
0	24.8 ± 1.5	6.4 ± 0.7	0.7 ± 0.1	0.8 ± 0.0	67.3
0.5	14.7 ± 1.1	3.2 ± 0.2	3.9 ± 0.3	0.7 ± 0.1	77.5
1	16.1 ± 1.1	2.3 ± 0.1	8.5 ± 0.6	0.7 ± 0.1	72.4
2	28.0 ± 0.8	4.0 ± 0.3	2.4 ± 0.3	0.4 ± 0.1	65.2
3	38.8 ± 1.3	7.1 ± 2.5	5.5 ± 0.5	0.3 ± 0.0	48.3
4	26.5 ± 1.5	5.6 ± 0.2	6.5 ± 0.4	0.3 ± 0.0	61.1
5	24.7 ± 1.1	3.5 ± 0.2	4.1 ± 0.3	0.5 ± 0.0	67.2

[§]Age-dependent changes in the fat body composition of the PL female crickets. Values (means ± S.E. of 20-30 determinations) are presented as % of the fat body wet weight contributed by the corresponding substance class. [#]Water content of the fat body was not determined experimentally, but calculated by subtracting percentage lipid, protein, glycogen and free carbohydrate content from 100%; the experimental data from the adult fat body indicates that the non-detectable substances contribute about 2 to 17% of the wet weight (Lorenz and Anand, 2004).

Table 18:   Correlation coefficient and significance of age-dependent changes in fat body composition[§]

Parameter	Correlation coefficient	Probability of error
weight (d0→d5)	0.9361	0
lipid (d0→d3)	0.8363	2.92×10^{-30}
lipid (d3→d5)	-0.1547	1.01×10^{-1} (ns)
protein (d0→d3)	0.7157	7.80×10^{-19}
protein (d3→d5)	-0.5043	4.27×10^{-6}
glycogen (d0→d4)	0.8199	4.30×10^{-33}
glycogen (d4→d5)	-0.3282	9.99×10^{-3}
free carbohydr. (d0→d5)	0.6437	2.15×10^{-20}

[§]Correlation coefficient and significance of age-dependent changes of fat body fresh weight, total lipid, protein, glycogen and free carbohydrate content as determined by Spearman rank-correlation. Means ± S.E. of 20 to 30 determinations; [ns]not significant.

4. Discussion

4.1. Regulation of energy metabolism by AKH

4.1.1. Effects of AKH on lipid synthesis and degradation

The accumulation of energy-rich substances during larval instars and the early adult stage is of prime importance for insects. Later in the adult stage, the energy stores are needed for energy-demanding processes like flight and reproduction (Beenakkers et al., 1985b). The fat bodies from the cricket, *Gryllus bimaculatus* show high lipogenic activity in *in vitro* acetate incorporation assay (Lorenz, 2001). The activity was tested in the incubation media MM199 and FBIM (Fig. 2). MM199 is a complex mixture of many chemicals, including amino acids and vitamins, in contrast to the FBIM. In some experiments it was necessary to manipulate calcium and acetate concentrations, hence FBIM was developed; this is a modified version of the fat body incubation buffer described by Gokuldas et al. (1988). The rate of acetate incorporation by the fat body of the cricket was higher when incubated in the incubation medium MM199 than in the FBIM. Since AKH significantly inhibited acetate incorporation into lipid by fat body in both media *in vitro* (Fig. 2), FBIM could be used as an alternative to MM199. The antibodies raised against AKH from *Pyrrhocoris apterus* do not recognize AKH from *G. bimaculatus* (Goldsworthy et al., 2002b), however Pyrap-AKH significantly inhibited lipid synthesis in the cricket fat body (this study).

The acetate provided in the incubation medium is used to build fatty acids, which are assembled to form mainly neutral lipids (Lorenz, 2001). The inhibition of acetate incorporation by AKH could be either at the level of fatty acid synthesis or in the coupling of fatty acid chains to glycerol. This question was addressed by using glycerol as a radiolabeled substrate instead of acetate (discussed later). In the presence of palmitic acid the fat body showed a tendency towards reduced acetate incorporation, most probably due to the feed-back inhibition of acetate incorporation into fatty acids and subsequently into neutral lipids (Table 1). Glycerol acts as a backbone for neutral lipid synthesis, hence it seems to have no effect on the fate of acetate. This is supported by the demonstration of acetate incorporation inhibition by AKH to the same extent both in the presence and in the absence of glycerol (Table 1).

In insects utilizing lipids as a fuel for flight, AKH activates hydrolysis of TAG and release in the form of *sn*-1,2-DAG from the fat body into the hemolymph (Lok and Van der Horst, 1980; Tietz and Weintraub, 1980; Arrese and Wells, 1997). The DAG is loaded on HDLp and transported to the muscle cells where lipophorin lipase hydrolyzes DAG into FAs and glycerol (Ryan and Van der Horst, 2000). The FAs are oxidized by the muscle cells while the glycerol is transported back to the fat body (Candy et al., 1997). On the initiation of flight activity in locust, the concentration of glycerol in the flight muscles rapidly elevates to six times the basal level, while in the fat body the glycerol concentration increases nearly 50-fold (Van der Horst et al., 1983). The concentration of glycerol in locust hemolymph increases six to ten-fold after about an hour's flight (Candy et al., 1976; Van der Horst et al., 1983). The incorporation of radiolabeled glycerol into lipid by the cricket fat body indicates that the fat body is able to reuse the glycerol released at the muscle cells (Table 2). AKH activated glycerol incorporation into lipid by several folds. Similar results obtained for locusts (Table 2) were in agreement with the earlier reports by Candy et al. (1976) and Van der Horst et al. (1983). This means the phenomenon is not limited to locusts. The increased glycerol incorporation in the presence of AKH can be due to the increased availability of fatty acid, as suggested by Candy et al. (1997) or by the activation of either glycerol kinase, glycerol 3-phosphate acyltransferase or other enzyme/s in the pathway, by AKH. However, external supply of fatty acid did not increase the rate of glycerol incorporation (Table 2; Candy et al., 1976). The activation of monoacylglycerol-acyltransferase seems less likely, as in *Manduca sexta*, injection with AKH did not stimulate the enzyme (Arrese et al., 1996).

Glycerol can be incorporated into lipid directly or, after hydrolysis, can be utilized for fatty acid synthesis. The incorporation of glycerol into trehalose by the fat body is also a major pathway for glycerol utilization (Candy et al., 1976; Van der Horst et al., 1983), which was not dealt in this study. The fate of glycerol incorporated into lipid was confirmed by using TLC and saponification. The separation of lipid extract into lipid classes by TLC showed that most of the glycerol was incorporated into TAG and *sn*-1,2-DAG (Table 3). Even though AKH activated glycerol incorporation into lipid, to large extent it did not influence the pattern of glycerol incorporation into lipid classes. Upon saponification most of the radioactivity was recovered in the aqueous phase, which means glycerol was incorporated into the lipid backbone and not in the fatty acids. The radioactivity recovered in the organic phase was due to incomplete saponification as confirmed by TLC. The inhibition of

palmitate incorporation into lipids by 10^{-8} M AKH in the fat body was about 14%, which is very low compared to 90 to 95% inhibition of the acetate incorporation. The distribution of radioactive palmitate incorporated into lipid classes was not drastically changed by the AKH treatment (Table 4). From the data on the regulation of glycerol incorporation into lipid by AKH, it is clear that AKH inhibits synthesis of fatty acid chain and not the coupling of fatty acids with glycerol backbone.

These results not only explain the lipid synthesis but also shed some light on the mechanism of lipid degradation for mobilization in crickets. None of the three mechanisms of *sn*-1,2-DAG synthesis from TAG, mentioned in the introduction (page 9), seem to explain this very important process completely. According to the MAG pathway, TAG is hydrolyzed into 2-MAG followed by acylation of 2-MAG (Chino and Gilbert, 1965; Tietz et al., 1975; Tietz and Weintraub, 1978). The MAG pathway has been shown to be present in the cockroach, *Periplaneta americana* (Hoffman and Downer, 1979), while locust fat body monoacylglycerol acyltransferase specifically acylates 2-MAG (Tietz et al., 1975). This pathway explains the process only partly, as it does not account for the glycerol released at the muscle cells; AKH has been shown to increase the flux of glycerol (Candy et al., 1976; Van der Horst et al., 1983). The *sn*-3-glycerophosphate pathway suggests the *de novo* synthesis of DAG from glycerol-3-phosphate via phosphatidic acid. The FAs released by TAG hydrolysis can be used for the acylation reactions (Arrese and Wells, 1994). The fat body TGL shows lowest activity towards 2-MAG compared to 1-MAG or 3-MAG (Arrese and Wells, 1994), implying 2-MAG as the major end product emerging from TAG hydrolysis. Therefore the *sn*-3-glycerophosphate pathway might not be the preferred pathway. However, the glycerol pumped back from muscle cells to the fat body can be acylated by this pathway. The stereospecific hydrolysis of TAG into *sn*-1,2-DAG seems to be the least probable pathway, as until now no lipase which can stereospecifically hydrolyze TAG into *sn*-1,2-DAG has been reported. Indeed, equal quantities of *sn*-1,2-DAG and *sn*-2,3-DAG are yielded on TAG hydrolysis by the fat body TGL (Arrese and Wells, 1994).

From the results of this study and earlier reports, a possible mechanism could be put forward: the release of AKH on initiation of flight instantaneously inhibits *de novo* fatty acid synthesis, which is a highly energy-demanding process in itself. The TGL is activated and/or translocated, which hydrolyzes TAG into 2-MAG and two FA molecules. 2-MAG is

acylated at position 1 to form *sn*-1,2-DAG and released into the hemolymph. It is transported to the muscle cells where FAs are released by the action of lipophorin lipase. The FAs are oxidized in the muscle, while glycerol is transported to the fat body. The glycerol can undergo glycerol-3-phosphate pathway to produce *sn*-1,2-DAG for release into the hemolymph. The FA leftover in the fat body upon TAG hydrolysis by TGL is used during the reacylation of the glycerol. The fact that AKH activates glycerol incorporation into lipid (Table 2), strengthens this hypothesis. The radiolabeled glycerol was recovered mostly in the TAG fraction (Table 3) because in the *in vitro* system used in this study, there was no possibility for the fat body to release *sn*-1,2-DAG. The requirement of hemolymph in the fat body incubation medium for lipid release has been demonstrated in locusts (Tietz, 1962). In the *in vivo* system *sn*-1,2-DAG is released into the hemolymph and quickly transported to the muscles for oxidation, maintaining relatively low concentrations in the fat body.

4.1.2. Regulation of enzymes of lipid metabolism by AKH

A number of enzymes are involved in the synthesis and mobilization of lipid in insects. AKH inhibits lipid synthesis and activates lipid degradation for mobilization. Therefore it was anticipated that AKH regulates the key enzyme/s involved in these processes. However, AKH treatment of the fat body had no consistent or significant effect on the ACCase (Table 5), ACL (Table 6) and FAS (Table 7) activities, even though the fat body is highly sensitive to AKH (Lorenz, 2001). It is possible that AKH might be inhibiting one or more enzymes of lipid synthesis that can be different from those studied here, for example malic enzyme. The effects of degradation of the enzymes by proteases in the tissue homogenate could also play a role. Another possibility could be that the fat body enzyme/s inhibited by AKH recover from the inhibition during the extraction and/or *in vitro* assay. Protein phosphatases are likely to change the activities of some enzymes; in mammals ACCase is inactivated by phosphorylation (Munday, 2002). AKH is believed to act in analogy to glucagon, which phosphorylates and inactivates ACCase (Munday, 2002). In mammals glucagon regulates FAS at transcriptional level (Goodridge, 1986; Goodridge et al., 1986).

The activity of fat body TGL in LL was relatively low compared to the adults (Fig. 3). During the early and late stages of the LL, TGL activity was higher than the period in between, suggesting the possible requirement of lipid for energy supply for development

and molting. In the adult stage, the gradual increase in the TGL activity right from the beginning of the stage seems to be necessary for the mobilization of lipid for egg development and/or for flying, as the adult female fat body mobilizes about 100 mg lipid between day 2 and 8 to support the growing oocytes (Lorenz and Anand, 2004). AKH significantly activated the fat body TGL in the adult crickets, whereas the effect was hardly significant in the LL (Fig. 3). Up to about 73% activation of the TGL was observed in the adults, which is in agreement with the reported activation of about 60% in locust (Ogoyi et al., 1998). In vertebrates, the enzyme responsible for the degradation of TAG is hormone-sensitive lipase (HSL) (Ryan and Van der Horst, 2000). It is activated *in vitro* by phosphorylation via cAMP-dependent protein kinase. The mechanism seems to involve both translocation of HSL from the cytosol to the lipid droplet (Egan et al., 1992; Morimoto et al., 2001) and conformational changes in the HSL molecule. The TGL purified from the *M. sexta* fat body is also phosphorylable by cAMP-dependent protein kinase (Arrese and Wells, 1994). Researchers have also been faced with difficulties in the provision of stable substrate for lipase in the *in vitro* assay system (Tietz and Weintraub, 1978). Considering all these factors, it is reasonable to believe that the level of activation of lipase by hormones can be much higher than that observed in the *in vitro* system.

AKH penetrates the cuticle of an insect when applied topically and elicits the effects observed on its injection (Kodrík et al., 2002; Lorenz et al., 2004). The topical application of AKH on adult crickets caused significant increase in the hemolymph lipid titers (Fig. 4). It increases locomotor activity of the cricket, probably by increasing the hemolymph lipid titers (Lorenz et al., 2004). Similar effect was observed in the flightless bug, *Pyrrhocoris apterus* (Kodrík et al., 2002). These effects are brought about most likely via the activation of TGL, however, TGL extracted from the fat bodies dissected from the insects topically applied with AKH showed no significant activation compared to the controls (Fig. 4). Again the inability to maintain the status of TGL during extraction and *in vitro* assay or its possible activation in the *in vivo* system by translocation to the lipid droplet can be responsible for these contradictory results. The situation is totally different in the flying insects, where the released lipid is quickly utilized. The inhibition of TGL from the locust was observed at higher substrate level (Ogoyi et al., 1998).

4.2. FAS and acetate incorporation activity

In the acetate incorporation assay, incorporation of radiolabeled acetate into fatty acids and subsequently into nonpolar lipids is monitored, whereas the spectrophotometric FAS assay relies on the measurement of the rate of NADPH oxidation during fatty acid synthesis. Thus, it is independent of the incorporation of fatty acids into nonpolar lipids. These two assays are therefore different from each other. However, the acetate incorporation and FAS activities showed high age-dependent correlation *in vitro* (Fig. 5). The activities were low immediately after adult emergence, then increased quickly and stayed at a high level during the initial period of the adult stage. After day 4, the activities declined quickly and stayed at basal levels thereafter. These results at least indicate, that age-dependent changes in lipid synthesis measured *in vitro* resemble the situation in the living animal. Furthermore, the high lipogenic activity in the initial phase of the adult stage led to a substantial increase in total lipid in the fat body (Lorenz and Anand, 2004). High lipogenesis that precedes the onset of vitellogenic oocyte growth has been shown for locusts (Gokuldas et al., 1988; Lee and Goldsworthy, 1995) and mosquitoes (Ziegler, 1997). Lipid reserves can also be built up to support flight. Flight activity in *G. bimaculatus* reared at 27°C starts on day 2-3 after adult emergence (Tanaka et al., 1999; Woodring et al., 2002), but almost exclusively trivial flights (few seconds to minutes) are observed. Thus, except for long distance flights, the required amount of energy to fuel flight activity in *G. bimaculatus* seems rather small compared to the energy a female needs to support egg production. In the wing polymorphic cricket, *Gryllus firmus*, however, the total amount of lipid that is accumulated during early adult life is lower in short-winged females that are not capable of flying but produce high egg numbers, compared to long-winged females that readily fly but produce fewer eggs (Zera and Larsen, 2001; Zhao and Zera, 2001). This indicates that larger lipid stores are built up to support flight activity rather than reproduction. On the other hand, the flightless morph synthesizes higher amounts of phospholipids that are important for egg-production. Thus, in this species, biochemical differences in lipogenesis underlie the phenomenon of polymorphism and high lipid synthesis is negatively correlated with reproduction (Zhao and Zera, 2002).

The pattern of the age-dependent changes in the acetate incorporation activity in the cricket fat body was comparable to that earlier reported by Lorenz (2001), although the highest activity was almost double in the current study (267.4 ± 30.8 nmol acetate incorporated/h/fat body). This difference might be due to several reasons. High animal to

animal and day to day variation as well as some seasonal variation in the rate of acetate incorporation has been observed (Lorenz, 2001; this study). To avoid inbreeding, crickets collected in wild are frequently added to the colony maintained in our laboratory, which could also contribute to the variation. An earlier effort to standardize the method using fat body suspension was unsuccessful for this insect (Lorenz, 2001).

4.3. Signal transduction of AKH

The signal transduction of AKH peptides has been studied in a few insects and for their selected effects only (Van der Horst et al., 1999; Gäde and Auerswald, 2003). Although, the first AKH was fully characterized long back in 1976 (Stone et al., 1976), it took more than 25 years to clone its receptor. The first AKH receptors cloned from the fruitfly *Drosophila melanogaster* and the silkworm *Bombyx mori* are G-protein-coupled receptors (Park et al., 2002; Staubli et al., 2002). They are structurally and evolutionarily related to the gonadotropin-releasing hormone receptors from vertebrates. The involvement of inositol 1,4,5-trisphosphate (IP_3) and Ca^{2+} is suggested in the signal transduction during the hypertrehalosemic effect of AKH in locusts, cockroaches, and beetles (Gäde and Auerswald, 2003), whereas during the hyperlipemic and/or hyperprolinemic effects cAMP and Ca^{2+} are the secondary messengers of AKH in locusts, moths and beetles (Gäde and Auerswald, 2003). However, nothing is known about the mechanism of action of AKH in the cricket, *G. bimaculatus* and about the mechanism leading to the inhibition of lipid synthesis in the insect fat body in general.

Hemolymph lipid titers increase when crickets are flown for 1 to 4 h and the injection of AKH into crickets induces an increase in the carbohydrate and lipid titers in the hemolymph (Woodring et al., 2002). The release of lipid is stronger and the lipid concentration in the hemolymph is higher than that of the carbohydrates (Woodring et al., 2002), indicating that lipid plays a more important role in metabolism in the cricket. Indeed, on days 0 to 4, about 50% of the wet weight of the fat body of the cricket is formed of lipid, compared to less than 1% of glycogen and free carbohydrate together (Lorenz and Anand, 2004). The use of trehalose during the initial and lipid during later stage of flight activity is suggested in the cricket (Tanaka et al., 1999). In a related species, *Gryllus assimilis*, lipid has been identified as a major flight fuel, which seems to be true for the whole genus *Gryllus* (Zera et al., 1999). Since lipid is the major fuel in crickets, efforts

were concentrated on the secondary messengers, cAMP and Ca^{2+}, which have been identified in the signal transduction of AKH during hyperlipemia. The characteristic of AKH to inhibit lipid synthesis in *G. bimaculatus* fat body *in vitro*, was used to study the signal transduction system, which is highly sensitive and faster compared to the activation of lipase.

4.3.1. Calcium

4.3.1.1. Extracellular calcium

The presence of extracellular Ca^{2+} (Ca_e^{2+}) is essential for the inhibition of acetate incorporation into lipid by AKH in the cricket fat body *in vitro* (Fig. 6). The requirement of Ca_e^{2+} for the AKH effect has also been reported in locusts, moths and beetles, where AKH stimulates influx and efflux of Ca^{2+} into/from fat body cells (Spencer and Candy, 1976; Van Marrewijk et al., 1991; Van Marrewijk et al., 1993; Arrese et al., 1999; Auerswald and Gäde, 2001). To confirm whether the entry of Ca_e^{2+} into the cytosol is necessary for the AKH effect in the cricket fat body, incubations were carried out in the presence of calcium chelators and calcium channel blockers. The Ca^{2+} chelators BAPTA, BAPTA-AM (a membrane-permeable form of BAPTA) and Quin 2-AM (also membrane-permeable) showed no effect on the AKH action on fat bodies (Table 8). Since the incubation medium itself contains 3 mM Ca^{2+}, the chelators (which were used at relatively lower concentrations) could not chelate Ca^{2+} completely. The use of higher concentrations of the chelators was not possible due to constraints of solubility and/or drastic reduction in the rate of acetate incorporation. The complete chelation or removal of Ca^{2+} from the incubation medium would prompt depletion of intracellular Ca^{2+} stores (Steele and Ireland, 1999). This would render AKH ineffective, if the AKH action depends on the intracellular Ca^{2+} stores. In the presence of the calcium channel blockers, nifedipine, verapamil, $LaCl_3$ or ruthenium red, AKH inhibited acetate incorporation into lipid (Table 9). This suggests that entry of Ca_e^{2+} into the cytosol is not necessary for the action of AKH, but the presence of Ca^{2+} in the incubation medium is essential, perhaps for the binding of AKH with its receptor. In fact, divalent ions are essential for the binding of AKH to the receptor in the membrane preparations in *M. sexta* (Ziegler et al., 1995).

4.3.1.2. Intracellular calcium

TMB-8 HCl, an intracellular calcium (Ca_i^{2+}) antagonist and dantrolene, inhibitor of Ca^{2+} release from sarcoplasmic reticulum via inhibition of ryanodine receptor channels, had no effect on acetate incorporation, as well they did not interfere with the AKH action (Table 10). The effect of AKH on the acetate incorporation was mimicked by thapsigargin, which releases Ca^{2+} from intracellular stores (Fig. 7). Thapsigargin mimicked the effects of AKH also in locusts and moths (Van Marrewijk et al., 1993; Arrese et al., 1999). Thapsigargin showed no inhibitory effect in absence of Ca_e^{2+}, most likely due to the prior depletion of intracellular calcium stores caused by the absence of Ca^{2+} in the incubation medium. The inhibitory effect of thapsigargin was not reversible because it inhibits sarco- endoplasmic reticulum Ca^{2+}-ATPases irreversibly causing permanent elevation of Ca_i^{2+} levels (Fig. 8). TMB-8 HCl failed to interfere with the action of thapsigargin and AKH as well, and does not seem to be an effective Ca_i^{2+} antagonist at least in the cricket fat body (Table 10, 11). In contrast, it reduced effect of AKH in the locust fat body (Van Marrewijk et al., 1993).

The Ca^{2+} ionophore A23187 significantly inhibited acetate incorporation only when the Ca_e^{2+} concentration was 3 mM or higher (Table 12). A similar effect was observed when fat bodies were treated with ionomycin, which is a more effective ionophore than A23187 (Fig. 9). This proves that the increase in the Ca_i^{2+} concentration in the fat body cells leads to the inhibition of acetate incorporation into lipid. The failure of TMB-8 HCl to interfere with the effect of ionomycin strengthens the argument of its ineffectiveness to act as a Ca_i^{2+} antagonist in this system (Table 13). The fat bodies incubated with high ionomycin concentration (50 µM) recovered completely from the inhibition of lipid synthesis when incubated in a medium without ionomycin (Fig. 10). It confirms that the effect of ionomycin on the fat body was specific and non-toxic.

Suramin activates release of Ca^{2+} from vertebrate skeletal muscle sarcoplasmic reticulum by activating ryanodine receptors. The inability of suramin to inhibit the acetate incorporation activity might be due to the lower concentration used or ineffectiveness in the insect fat body cells (Fig. 11).

4.3.1.3. Influx and efflux studies: effects of AKH

The treatment of the fat body with AKH had no effect on the influx of acetate and Ca^{2+} into the cells (Table 14). Acetate seems to move across the cell membrane freely, as its incorporation into lipid starts immediately, without a measurable lag-phase (Lorenz, 2001). Most probably, the intracellular concentration of acetate is equal to its concentration in the incubation medium. In contrast, the cytosolic concentration of Ca^{2+} is strictly controlled. The efflux of Ca^{2+} from the fat body cells was also not influenced by AKH (Table 15). The results from the Ca^{2+} channel blockers, Ca^{2+} ionophores, influx and efflux studies support the idea that entry of Ca_e^{2+} into the cells is not involved in the signal transduction of AKH. The magnitude of the increase in the Ca_i^{2+} concentration would be much higher had a capacitative entry of Ca_e^{2+} into the cytosol taken place. The release of Ca^{2+} from intracellular Ca^{2+} stores might increase the Ca_i^{2+} concentration to smaller extent, hence does not seem to warrant regulation by AKH as indicated in other insects.

4.3.1.4. Effect of caffeine, theophylline, thapsigargin and ionomycin on TGL activity

Caffeine and theophylline inhibit the adenosine 3',5'-cyclic monophosphate (cAMP) phosphodiesterase and increase cAMP levels in the cells. In addition, caffeine affects the cellular calcium levels by releasing calcium from the intracellular stores (Kim et al., 1997). Caffeine, theophylline, thapsigargin and ionomycin mimic AKH in two different assays, indicating a similar mode of action. They either activated or showed a tendency towards activation of fat body TGL, with the exception of 5 µM ionomycin at 3 mM Ca_e^{2+} (Fig. 12) and inhibited acetate incorporation into lipid by the cricket fat body (the effects of caffeine and theophylline are discussed in the following section in detail). Theophylline translocates HSL in the fat cells of the rat (Morimoto et al., 2001).

4.3.2. cAMP

4.3.2.1. Effects of caffeine and theophylline on acetate incorporation

Caffeine and theophylline significantly inhibited acetate incorporation into lipid by the fat body (Fig. 13), which was independent of the presence of Ca^{2+} in the incubation medium (Fig. 14). The reversible nature of the inhibitory effect shows that it was specific and non-toxic (Fig. 15). Caffeine in combination with AKH shows an additive but not synergistic effect on the acetate incorporation by the fat body (Fig. 16). The effect of AKH was countered by ryanodine, an inhibitor of Ca^{2+} release from sarcoplasmic reticulum (Fig. 17), whereas the effect of caffeine was not antagonized by ryanodine, but rather increased. This indicates that the mode of the action is not completely similar for AKH and caffeine.

4.3.2.2. Effects of cAMP analogues, cAMP agonists and AKH on acetate incorporation and intracellular cAMP levels

The cell-permeable cAMP analogues, 8-Br-cAMP, 8-CPT-cAMP, Sp-5,6-DCI-cBIMPS, or db-cAMP together with 1 mM IBMX (a phosphodiesterase inhibitor) had no effect on the acetate incorporation by the cricket fat body (Fig. 18), suggesting that cAMP is not involved in the signal transduction of AKH. However, forskolin showed a tendency to inhibit acetate incorporation in presence of IBMX, while cholera toxin significantly inhibited the process (Table 16). Forskolin and cholera toxin increase intracellular cAMP levels by activating adenylate cyclase. IBMX alone also showed a tendency for inhibition, but this effect was not significant. Thus, the results were confusing, making it difficult to assess the role of cAMP in the inhibition of lipid synthesis. To decipher the mechanism, the intracellular cAMP levels in the fat body were measured after treatments with forskolin, IBMX and AKH (Fig. 19). A multifold increase in the intracellular cAMP concentrations was observed on incubation of fat bodies with IBMX. The effect was augmented in the presence of forskolin. Since IBMX and forskolin did not inhibit acetate incorporation, even though they increased the intracellular cAMP concentrations, cAMP seems to be not involved in the inhibition of acetate incorporation. Indeed, AKH treatment of the fat bodies had hardly any effect on the intracellular cAMP levels. Caffeine seems to act via the release of Ca^{2+} from intracellular stores and not via increase in the cellular cAMP concentrations. The involvement of cAMP in the AKH signal transduction was previously indicated in the beetle, *P. sinuata*, where cAMP levels in the fat body increase by 6 to 7 folds upon injection with AKH (Auerswald and Gäde, 2000).

4.3.3. Diacylglycerol

In the phosphoinositide cascade, phospholipase C hydrolyzes phosphatidyl inositol 4,5-bisphosphate to produce two messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol. Diacylglycerol activates protein kinase C (PKC), which phosphorylates many target proteins. This property is imitated by phorbol esters because of their resemblance to diacylglycerol. The activation of PKC by PMA (a phorbol ester) had no effect on the acetate incorporation activity, suggesting that DAG is not a messenger in the inhibition of the activity (Fig. 20). However, the possible role of IP_3 in the signal transduction can not be ruled out. The involvement of IP_3 during hypertrehalosemic effect is well known (Steele et al., 2001; Auerswald and Gäde, 2002; Gäde and Auerswald, 2003).

4.3.4. Tentative overview of AKH signal transduction in *G. bimaculatus*

There is a marked difference in the signal transduction of AKH in the cricket compared to the reported mechanisms in other insects (Gäde and Auerswald, 2003). In the cricket, AKH seems to bind to a G-protein-coupled receptor on the cell membrane, which is facilitated by Ca^{2+}. The receptor changes conformation and interacts with a GTP-binding protein, which in turn transduces the signal to an enzyme that produces a second messenger in the cytoplasm and thereby amplifies the message. The secondary messenger leads to the release of Ca^{2+} from the intracellular calcium stores, which causes the activation of TGL and inhibition of fatty acid synthesis. From this study it is still not clear how Ca^{2+} brings about these effects, a point that needs further investigation. The signal transduction was studied using mainly the process of acetate incorporation and therefore warrants caution in applying the results to other effects of AKH.

4.4. Penultimate larval instar crickets

During the larval stages insects tend to build up huge energy stores, which are used during the adult stage. The physiological requirements for males and females are different during the adult stage, but they seem to be similar during the larval stages. The pattern of the acetate incorporation activity was very similar both in the male and female penultimate larval instar cricket fat bodies (Fig. 21). The activity was low at the beginning and at the end of the instar, which is true also for the last larval instar and adult stage (Anand and Lorenz, 2003). The lipogenic activity of the PL cricket fat body was significantly inhibited by AKH throughout almost the entire duration of the instar (Fig. 21), implying that AKH receptors are expressed as early as this stage. The inhibition by AKH was dose dependent with the EC_{50} of 7.9 x 10^{-11} M AKH (Fig. 22). The PL fat body showed intermediate sensitivity to AKH in terms of EC_{50} compared to the adult and LL fat body, which showed the highest and lowest sensitivities respectively (Lorenz, 2001; Anand and Lorenz, 2003). In stark contrast to the adult and LL crickets, in which the lowest concentrations of AKH required for the significant inhibition of the acetate incorporation activity were 5 x 10^{-11} and 10^{-9} M respectively, it was only about 10^{-12} to 10^{-14} M in the PL crickets. Thus, minute quantities of AKH in the hemolymph, which might not inhibit lipid synthesis in the adult and LL crickets, would be enough for the inhibition in the PL crickets.

4.4.1. Fat body composition

The development of the larval instars of the cricket depends on both temperature and diet (Merkel, 1977). It is faster at higher temperatures and it is further accelerated by higher protein content of the diet. The physiological development of an insect can be understood by the analysis of its fat body, which is the major center of energy storage and metabolism. The composition of the fat body of the female PL cricket was age-dependent (Fig. 23). During the initial phase of the larval instar, lipogenic activity is very high, which is followed by the peaking of the lipid and protein content of the fat body. The lipid and protein content of the fat body reached almost 46% of the wet weight of the fat body on day 3 compared to the 9.2% of glycogen and free carbohydrate together on day 1 (Table 17).

Remarkably, the PL cricket fat body contains glycogen and free carbohydrate in much higher proportions compared to the adult fat body, where both together contribute just around 1% of the wet weight (Lorenz and Anand, 2004). A huge amount of lipid is synthesized and stored by the cricket during the development, as the highest lipid content

of the PL fat body is about 10 mg, that increases to about 50 mg in the adult stage (Lorenz and Anand, 2004; this study); similarly, an about a three fold increase in the protein content is observed (from about 2 mg to about 6 mg). In contrast, the highest glycogen content of the larval fat body is about 2 mg, that decreases to about half a milligram in the adult stage. The free carbohydrate content of the fat body is minute during both the stages (less than half a milligram) and hence does not seem to have significant role in the energy storage. This indicates that there is a major shift in the physiology, where carbohydrates seem to have a more important role during the larval stage than in the adult stage. Similar conclusion can be drawn from the results in *L. migratoria* (Van Marrewijk et al., 1984) and *M. sexta* (Ziegler, 1984; Ziegler et al., 1990). The importance of differential allocation of energy resources is indicated by the difference in the lipid classes and storage tissues in a wing-polymorphic cricket, *Gryllus firmus* (Zhao and Zera, 2002).

4.5. Role of AKH in physiology and reproduction

AKH preferentially inhibits vitellogenin synthesis and possibly plays a role in insect reproduction (Moshitzky and Applebaum, 1990). Frequent injection of AKH elicits deleterious effect on egg production by inhibiting lipid and protein storage in the fat body (Lorenz, 2003). The storage of lipid in the fat body and its mobilization is essential during egg development in insects (Ziegler and Ibrahim, 2001; Lorenz and Anand, 2004). During vitellogenesis huge amounts of lipid are synthesized and mobilized (Lorenz and Anand, 2004), which poses the question, how AKH regulates both the processes simultaneously? The presence of AKH is essential for the mobilization of lipid stores, which would inhibit lipid synthesis. This problem might be solved by different sensitivities for AKH, or by carrying out the processes at different times. AKH concentrations in CNS (brain + CC + CA) and hemolymph show diel changes in the flightless bug, *P. apterus* (Kodrík et al., 2003), which is correlated with the diel rhythm in locomotory activity (Maxová et al., 2001). The cricket, *G. bimaculatus* shows 2 to 3 times higher locomotor activity during scotophase compared to that during photophase (Lorenz et al., 2004). This effect is brought about most probably by the increase in the hemolymph lipid titers (Lorenz et al., 2004), suggesting a possible diel rhythm of AKH release in the cricket as well. AKH is released upon starvation in larval instar locusts, but not in adults (Cheeseman and Goldsworthy, 1979; Candy, 2002). Upon starvation the lipid concentration increases in adult locusts (Candy, 2002) and moths (Ziegler, 1991), which is independent of AKH. Increase in the

hemolymph AKH concentration is also observed on poisoning or on initiation of flight (Candy, 2002). In conclusion, AKH is involved not only in lipid mobilization during flight, but seems to have an important function in developmental and reproductive processes, which have not yet been explored.

4.6. Outlook

A lot of work has been done on insect physiology and endocrinology, but many questions still remain unanswered. Insect physiologists have worked on selected insects and insect species, in spite of the enormous diversity not only in terms of number of insect species, but also in the variety of biochemical mechanisms they possess. In addition to their benefits as model systems, it is very important to know about the regulation of important processes in insect's life. With the evident hazardous and unspecific effects of the chemical pesticides, for which insects quickly develop resistance, the time has come to alter our pest management strategies. Neurohormones play very important roles in many fundamental processes in insects and could offer us an invaluable tool in insect pest management (Couillaud and Peypelut, 1995; Altstein, 2003; Gäde and Goldsworthy, 2003). Until now, efforts in this direction have not been very successful, but with improving research techniques and increasing knowledge of insect biochemistry, physiology, and endocrinology, success might be around the corner.

5. Summary

- The fat body from the cricket, *Gryllus bimaculatus* incorporates acetate, glycerol or palmitate into lipid *in vitro*.

- Adipokinetic hormone (AKH) inhibits acetate incorporation into lipid by the fat body from adult crickets *in vitro*. AKH inhibits palmitate incorporation into lipid to a small extent, however, it does not influence the incorporation of palmitate into different lipid classes.

- In the presence of AKH, glycerol incorporation into lipid by the adult cricket fat body increases by several fold. AKH does not influence the incorporation of glycerol into different lipid classes. The fat body incorporates glycerol mainly into triacylglycerol (TAG) and almost exclusively into the backbone.

- AKH inhibits fatty acid (FA) synthesis but not the coupling of FAs with the glycerol backbone.

- Triacylglycerol lipase (TGL) from the fat body, in last larval instar and adult crickets, shows an age-dependent pattern of activity. AKH activates TGL, but does not regulate ACCase, ACL and FAS upon treatment of the fat body for a short period, under the assay conditions. The FAS and acetate incorporation activities are highly correlated in an age-dependent manner and are highest on day 2 of the adult stage.

- The activation of the formation of *sn*-1,2-DAG from TAG, in the fat body by AKH seems to be via the removal of FAs at positions 1 and 3, followed by reacylation of 2-MAG.

- The presence of Ca^{2+} in the incubation medium is crucial for the inhibition of acetate incorporation by AKH; possibly it is essential for the binding of AKH to the receptor. The influx of acetate and Ca^{2+} into and efflux of Ca^{2+} from the cytosol are not affected by AKH.

- The release of Ca^{2+} from intracellular Ca^{2+} stores in the fat body, caused by thapsigargin, inhibits acetate incorporation irreversibly and shows a tendency for the activation of TGL.

- The Ca^{2+} ionophore ionomycin inhibits acetate incorporation by the fat body reversibly and shows a tendency for TGL activation. Another ionophore, A23187 also inhibits acetate incorporation.

- Caffeine and theophylline inhibit acetate incorporation by the fat body in a reversible manner and tend to activate TGL. Caffeine seems to act via the release of Ca^{2+} from intracellular stores and not via increase in the cellular cAMP concentrations.

- cAMP analogues and agonists do not influence acetate incorporation, however, the agonists, IBMX and forskolin cause a multifold increase in the cAMP concentrations in the fat body. AKH does not affect the cAMP concentrations in the fat body suggesting that cAMP is not involved in the signal transduction.

- As the activation of protein kinase C by PMA (a phorbol ester) does not affect acetate incorporation, diacylglycerol does not seem to be involved in the AKH signal transduction.

- The activation and/or translocation of TGL and inhibition of fatty acid synthesis by AKH seems to be via the release of Ca^{2+} from intracellular calcium stores.

- Lipid and protein form a major part of the fat body in the penultimate larval instar crickets, while glycogen forms a minor part. However, in comparison with the adult (glycogen content of fat body about 1%) and last larval instar crickets (glycogen content about 3%), the penultimate larval instar crickets contain higher amounts of glycogen (about 9%).

- AKH inhibits acetate incorporation into lipid by the fat body from penultimate larval instar crickets. The patterns of acetate incorporation and inhibition by AKH are similar in both, males and females. The inhibition is dose-dependent with an EC_{50} of 7.9 x 10^{-11} M AKH.

- AKH seems to play an important role in the development and reproduction of insects, in addition to its role during flight metabolism.

6. Zusammenfassung

- Der Fettkörper von *Gryllus bimaculatus* baut *in vitro* Acetat, Glycerin und Palmitinsäure in Lipide ein.

- Das Adipokinetische Hormon (AKH) hemmt die Inkorporation von Acetat in Lipide im Fettkörper adulter Grillen *in vitro*. AKH hemmt auch die Inkorporation von Palmitinsäure, allerdings nur sehr schwach. Es hat keine Auswirkungen darauf, in welche Lipidklassen Palmitinsäure eingebaut wird.

- AKH erhöht den Einbau von Glycerin in Lipide um ein Vielfaches, hat jedoch keinen Einfluss darauf, in welche Lipidklassen Glycerin eingebaut wird. Glycerin wird hauptsächlich in Triacylglycerine eingebaut und hier praktisch ausschließlich in den Glycerinanteil.

- AKH hemmt die Fettsäuresynthese, aber nicht den Einbau der Fettsäuren in Glycerine.

- Die Fettkörpertriacylglycerin-Lipase aus letzten Larven und adulten Grillen zeigt einen altersabhängigen Aktivitätsverlauf. AKH aktiviert die Triacylglycerin-Lipase *in vitro*, hat aber, zumindest unter den gewählten Versuchsbedingungen, keinen Einfluss auf die Aktivität der Acetyl-CoA-Carboxylase, der ATP-Citrat-Lyase und der Fettsäuresynthase. Zwischen dem altersabhängigen Verlauf der Aktivität der Fettsäuresynthase und dem Acetateinbau in Lipide besteht eine hohe Korrelation; die höchsten Werte werden an Tag 2 des Adultstadiums erreicht.

- AKH aktiviert die Bildung von *sn*-1,2-Diacylglycerin aus Triacylglycerin. Wahrscheinlich werden die Fettsäuren an Position 1 und 3 des Glycerins entfernt und anschließend das 2-Monoacylglycerin reacyliert.

- Das Vorhandensein von Ca^{2+} im Inkubationsmedium ist essentiell für die hemmende Wirkung des AKH auf den Acetateinbau in Lipide, und hier wohl insbesondere für die Bindung von AKH an seinen Rezeptor. Der Einstrom von Acetat und Ca^{2+} in die Fettkörperzellen, sowie der Ausstrom von Ca^{2+} werden nicht von AKH beeinflusst.

- Die durch Thapsigargin hervorgerufene Freisetzung von Ca^{2+} aus interzellulären Ca^{2+}-Speichern des Fettkörpers führt zu einer irreversiblen Hemmung des Acetateinbaus in Lipide und fördert tendenziell die Aktivität der Triacylglycerin-Lipase.

- Der Ca^{2+}-Ionophor Ionomycin hemmt den Acetateinbau reversibel und führt zu einer tendenziellen Aktivierung der Triacylglycerin-Lipase. Auch der Ca^{2+}-Ionophor A23187 hemmt den Acetateinbau.

- Koffein und Theophyllin hemmen den Acetateinbau in Lipide reversibel und erhöhen die Aktivität der Triacylglycerin-Lipase tendenziell. Koffein wirkt offenbar nicht über die Erhöhung der cAMP-Konzentration, sondern über die Freisetzung von Ca^{2+} aus intrazellulären Ca^{2+}-Speichern.

- cAMP-Analoge und -Agonisten hemmen den Acetateinbau in Lipide nicht, obwohl IBMX und Forskolin zu deutlich erhöhten cAMP-Titern im Fettkörper führen. Gegen eine Beteiligung des cAMP-Systems spricht auch die Tatsache, dass AKH die Konzentration von cAMP im Fettkörper nicht beeinflusst.

- Auch die Aktivierung der Proteinkinase C durch den Phorbolester PMA hat keinen Einfluss auf den Acetateinbau in Lipide. Daher scheinen auch Diacylglycerine nicht and der Signaltransduktion von AKH beteiligt zu sein.

- Die Aktivierung und/oder Translokation der Triacylglycerin-Lipase sowie die Hemmung der Fettsäuresynthese durch AKH scheint über die Freisetzung von Ca^{2+} aus intrazellulären Speichern vermittelt zu werden.

- Lipide und Proteine sind die vorherrschenden Inhaltsstoffe im Fettkörper von vorletzten Larven. Glycogen spielt als Speicherstoff nur eine untergeordnete Rolle; der Glycogengehalt im Fettkörper ist aber im Vergleich zu adulten Grillen (ca. 1%) und letzten Larven (ca. 3%) in den vorletzten Larven deutlich höher (ca. 9%).

- Der altersabhängige Einbau von Acetat in Lipide im Fettkörper von vorletzten Larven ist bei Weibchen und Männchen nahezu gleich, ebenso die Hemmung des Acetateinbaus durch AKH. Die hemmende Wirkung ist dosisabhängig, mit einer EC_{50} von 7.9×10^{-11} M AKH.

- Neben seiner regulierenden Funktion bei Aktivierung des Flugstoffwechsels scheint AKH auch eine wichtige Rolle bei der hormonellen Regulation von Entwicklung und Fortpflanzung der Insekten zu spielen.

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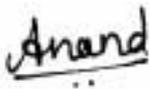
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Hiermit erkläre ich, dass ich die Arbeit selbständig verfasst und keine anderen als die von mir angegebenen Quellen und Hilftsmittel benutzt habe.

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