

cAMP-dependent protein kinase of *Manduca sexta* phosphorylates but does not activate the fat body triglyceride lipase

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Abstract

cAMP-dependent-protein kinase (PKA) is a central player of the adipokinetic signal that controls the mobilization of stored lipids in the fat body. Previous studies showed that adipokinetic hormone (AKH) rapidly activates PKA from the fat body of *Manduca sexta* (Arrese et al. (J. Lipid. Res. 40(3): 556)). As a part of our investigation on lipolysis in insects, here we report the purification and characterization of the catalytic subunit of PKA from the fat body of *M. sexta* and its role in the direct activation of the TG lipase in vitro. PKA was purified to apparent homogeneity and the identity of the protein was confirmed by MALDI-TOF and Western blot analysis. The enzyme showed a high affinity for Mg-ATP ($K_m = 39 \mu\text{M}$) and Kemptide ($K_m = 31 \mu\text{M}$) and was strongly inhibited by the PKA specific inhibitors PKI 5-24 and H89. *Manduca sexta* PKA only recognized serine residues as phosphate acceptor; theronine or tyrosine containing peptides were not phosphorylated. Purified fat body TG-lipase proved to be a good substrate of the purified kinase. However, phosphorylation of the lipase did not enhance the lipolytic activity of the enzyme in vitro. These results suggest that, besides lipase phosphorylation, the mechanism of AKH-induced activation of the lipolysis requires the involvement of other proteins and/or signals.

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1. Introduction

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

non-feeding pupal and adult periods, the TG stores decline (Ziegler, 1991).

Utilization of the fatty acids stored in the fat body requires hydrolysis of TG in a reaction catalyzed by a TG-lipase. Unlike vertebrates, in which stored fatty acids are mobilized as free fatty acids (FFA), a great number of insects mobilize fatty acids as diacylglycerol (DG) (Beenackers et al., 1985). Lipolysis is regulated by adipokinetic hormone (AKH) (Orchard, 1987; Van der Horst, 2003). The sequence of events leading to the stimulation of lipolysis induced by AKH still is not well understood (Gäde and Auerswald, 2003). The AKH receptors from the fruit fly *Drosophila melanogaster* and the silkworm *Bombyx mori* have been recently identified (Hauser et al., 1997; Staubli et al., 2002). These receptors are related to the mammalian gonadotropin

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

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

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

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

2. Materials and methods

2.1. Insects

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

2.2. Materials

$[\gamma\text{-}^{32}\text{PO}_4]\text{ATP}$ was purchased from ICN Biomedical (Irvine, CA). cAMP, leupeptin, aprotonin, Triton X-100, benzamidine, bovine PKA catalytic subunit, histone II AS, all peptide substrates and inhibitors were obtained from Sigma (St Louis, MO). DEAE-cellulose (DE-52) and P81 phosphocellulose filter paper were purchased from Whatman (Hillsboro, OR). Q-Sepharose was purchased from Amersham Pharmacia (Piscataway, NJ). Anti-human catalytic subunit of PKA antibody was obtained from Upstate Biotechnology (Lake Placid, NY). All other chemicals were of analytical grades.

2.3. Measurement of A-kinase activity

A-kinase activity was measured as described previously (Arrese et al., 1999). The final assay volume of 0.1 ml contained 50 mM MOPS (3-(N-morpholino) propane-sulfonic acid), pH 7.0, 10 mM magnesium acetate, 0.5 mM EDTA (ethylenediaminetetraacetic acid), 600 μM histone II AS, 0.2 mM $[\gamma\text{-}^{32}\text{PO}_4]\text{ATP}$ (1.5×10^6 cpm/nmole) and 10 μM cAMP when required. After incubation at room temperature for 15 min, the reaction was terminated by the addition of 5 μl of 6 N HCl. Seventy μl of the reaction mixture was spotted onto a disc of phosphocellulose filter paper (2.5 cm), and the filters were washed for 20 min in 50 mM NaCl four times (Roskoski, 1983). The radioactivity associated with the dried filters was counted by liquid scintillation counter using a Packard Tricarb 1900 TR. Under these conditions the kinase activity was linear up to the addition of 0.5 mg of total protein in the incubation mixture. Kinase activity was expressed in nanomol of phosphate transferred to histone per minute.

When synthetic peptide was used as phosphate acceptor for the purified kinase, the reaction mixture contained 50 μM peptide substrate, 50 mM MOPS, pH 7.0, 0.5 mM magnesium acetate, 0.25 mM EDTA, and 0.2 mM $[\gamma\text{-}^{32}\text{PO}_4]\text{ATP}$ (0.5×10^5 cpm/nmole).

2.4. Purification of *M. sexta* PKA catalytic subunit

All steps were carried out on ice or at 4 °C. Fat body tissue from 100 insects was collected in homogenizing

buffer (20 mM Tris, pH 7.4, 0.25 M sucrose, 1 mM EDTA, 0.1 mM benzamidine, 10 mg/l leupeptine, 1 mg/l aprotinin and 0.1% (v/v) 2-mercaptoethanol). The tissue was homogenized at a ratio of 3 ml per fat body using a Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was subjected to ultracentrifugation at $100,000 \times g$ for 1 h and the soluble extract was collected. The soluble extract was loaded onto a DE-52 column (3×10 cm) equilibrated with buffer A (50 mM Tris-HCl, pH-7.4, 1 mM EDTA, 0.1% 2-mercaptoethanol). The column was washed with 5 volumes of buffer A and developed with a linear salt gradient from 0–400 mM NaCl in buffer A. Fractions of 8 ml were collected and those containing PKA activity (eluted between 110–200 mM NaCl) were pooled, dialyzed against buffer A containing 30 mM NaCl and subjected to a second DE-52 column (2×5 cm) equilibrated with buffer A containing 30 mM NaCl. After extensive wash of the column with equilibration buffer, a solution of 1 mM cAMP in buffer A was passed through the column. Fractions containing kinase activity were pooled and the concentration of NaCl was increased to 50 mM and loaded onto a Q-sepharose anion exchanger column (2×1 cm) equilibrated with buffer A containing 50 mM NaCl. Under this condition the majority of kinase activity did not bind to the resin and was found in the flow trough. These fractions were collected, pooled and stored on ice until further use. For prolonged storage, the enzyme activity was preserved for several months at -20°C in the presence of 50% glycerol.

2.5. Other methods

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

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

For the Peptide Mass Fingerprinting, purified PKA catalytic subunit was separated on 10% SDS-PAGE and the band that was visualized by Coomassie staining was excised, minced and destained using 100% acetonitrile, followed by four washes in 1 ml water. The gel pieces were incubated for 20 min in $500 \mu\text{l}$ of 100 mM

ammonium bicarbonate followed by 20 min incubation with $500 \mu\text{l}$ of 50% acetonitrile in 50 mM ammonium bicarbonate. Gel pieces were dried under vacuum, rehydrated and digested with $50 \text{ ng}/\mu\text{l}$ trypsin (sequencing grade, Promega, Madison, WI) in 25 mM ammonium bicarbonate at 4°C overnight. Peptides were extracted and analyzed by MALDI-TOF mass spectrometry at the Core Facility of our department. Mass characterizations were performed using α -cyano-4-hydroxycinnamic acid as matrix, using external standards as calibrants. MSDB database was used to identify the peptides.

Phosphorylation of TG-lipase by PKAc: Fat body TG-lipase was purified as described previously (Arrese and Wells, 1994). TG-lipase ($7 \mu\text{g}$) was incubated in a final volume of 0.1 ml containing 50 mM MOPS, 1 mM magnesium acetate, 0.5 mM EDTA, 1 mM dithiothreitol, purified PKAc (0.25 units) and 0.2 mM $[\gamma\text{-}^{32}\text{PO}_4]\text{ATP}$ (5×10^6 cpm/nmol). After 15 min incubation at room temperature, the reaction was stopped by addition of electrophoresis sample buffer and the sample was analyzed by SDS-PAGE. Dried gels were then exposed to X-ray film. Control incubations in which purified PKAc was omitted indicated the absence of PKA activity, or other kinase activators, in the preparation of TG-lipase. On the other hand, when purified PKAc was incubated with $[\gamma\text{-}^{32}\text{PO}_4]\text{ATP}$ neither phosphorylation of PKAc (autophosphorylation) nor any other phosphorylation of endogenous substrates was observed. Unlabeled phosphorylated TG-lipase was made using the same conditions, except unlabeled ATP was used and the reaction was stopped by addition of 5 mM EDTA. Lipase activity of the phosphorylated and non-phosphorylated (control) form of the enzyme was measured using micellar $[\text{H}^3]\text{-triolein}$ as described previously (Arrese and Wells, 1994).

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

2.6. Statistics

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

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

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

^aThe enzyme activity was assayed in the presence of 10 μ M cAMP.

3. Results and discussion

3.1. Purification of *M. sexta* PKA catalytic subunit

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

cAMP-dependent protein kinase (PKA) is composed of two catalytic subunits and two regulatory subunits. The holoenzyme is inactive. Upon cAMP binding, the enzyme activates by releasing the catalytic subunits (Taylor et al., 2004). The large difference between the isoelectric points of the free catalytic (basic) and the regulatory subunit (acidic) has been used to facilitate the purification of the catalytic subunit from vertebrate tissues (Reimann and Beham, 1983). Using differential binding capacity on anion exchanger resin we developed a procedure for the purification of the catalytic subunit of PKA from the insect fat body (Table 1). Preliminary studies indicated that the vast majority of PKA is associated with the cytosolic fraction which is obtained as the soluble material after centrifugation ($100,000 \times g$) of fat body homogenate and removal of the fat cake. This material was subjected to DE-52 column chromatography. A linear concentration gradient of NaCl was used to elute the proteins from the column. Kinase activity inducible by cAMP was monitored in individual fractions by measuring the activity in the presence and absence of cAMP. Up to 400% increase of kinase activity induced by cAMP was found in fractions 16–35 (110–220 mM NaCl) indicating that PKA holoenzyme was eluting in this region as a single peak (Fig. 1). These fractions were pooled, dialyzed and subjected to a second DE-52 chromatography. After extensive wash, the catalytic subunit of PKA was dissociated and eluted from the column using 1 mM cAMP.

The kinase activity eluted as a very sharp peak but was not pure (Fig. 2, lane 2). To remove the

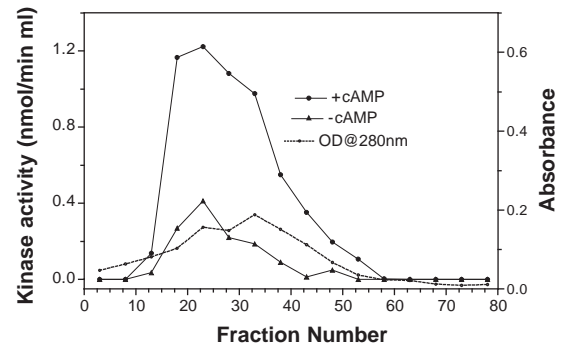


Fig. 1. Elution profile of PKA from DE-52(I) column. The soluble extract after $100,000 \times g$ centrifugation of adult *M. sexta* fat body homogenate was loaded into the column. The column was developed with 0–400 mM linear sodium chloride gradient in buffer A. Aliquots from fractions were assayed for A-kinase activity in presence (—●—) and absence (—▲—) of 10 μ M cAMP. Absorbance was monitored at 280 nm (—○—) of individual fractions.

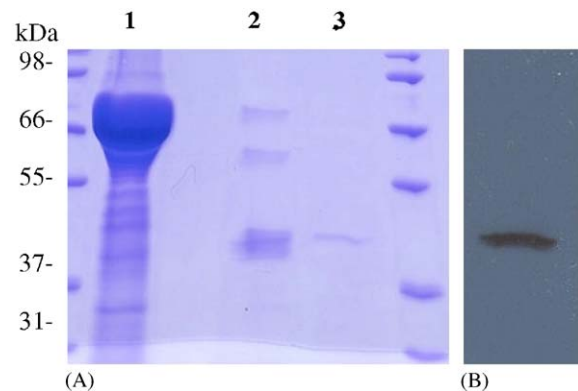


Fig. 2. SDS-PAGE and Western blot analysis of purified PKA. (A) Proteins were resolved in 10% acrylamide gel and stained with Coomassie Brilliant Blue. Lanes: (1) soluble extract; (2) after DE-52 (II); (3) after Q-Sepharose. (B) Western blot analysis of final preparation using human anti-catalytic subunit.

contaminants a strong anion exchanger, Q-Sepharose, was used. This was based on the observation that proteins co-eluting with PKA in DE-52 chromatography bind to the Q-Sepharose resin equilibrated in buffer containing 50 mM NaCl, while catalytic subunit of PKA does not bind. The majority (75%) of kinase activity was

found in the flow through having a specific activity of 266 nmoles/mg-min (Table 1).

SDS-PAGE analysis showed that this preparation contained a single protein band with an apparent molecular mass of 45.1 ± 0.2 kDa (Fig. 2A, lane 3). Similar sizes have been reported for the catalytic subunits of PKA from *Drosophila melanogaster* (Foster et al., 1984; Haracksa and Udvardy, 1992), the gall insects *Epiblema scudderiana* and *Eurosta solidaginis* (Pfister and Storey, 2002), and other animals (Taylor et al., 1990; Mehrani and Storey, 1995; Brooks and Storey, 1996). Moreover, Western blot analysis of the final preparation using polyclonal antibodies against

human catalytic subunit of PKA recognized this protein band (Fig. 2B).

The identity of the protein was confirmed further by trypsin fragmentation followed by MALDI-TOF analysis (Fig. 3).

The search for matching peptides identified 14 peptides that coincided precisely to the *Drosophila melanogaster* catalytic subunit of cAMP-dependent protein kinase (C31751) (Fig. 4). The correlation of the match was significant and the peptide matches covered 31% of the drosophila sequence. Further NCBI Blast search of individual peptides gave 97% identity to *Anopheles gambiae* and *Apis mellifera* and 88% to

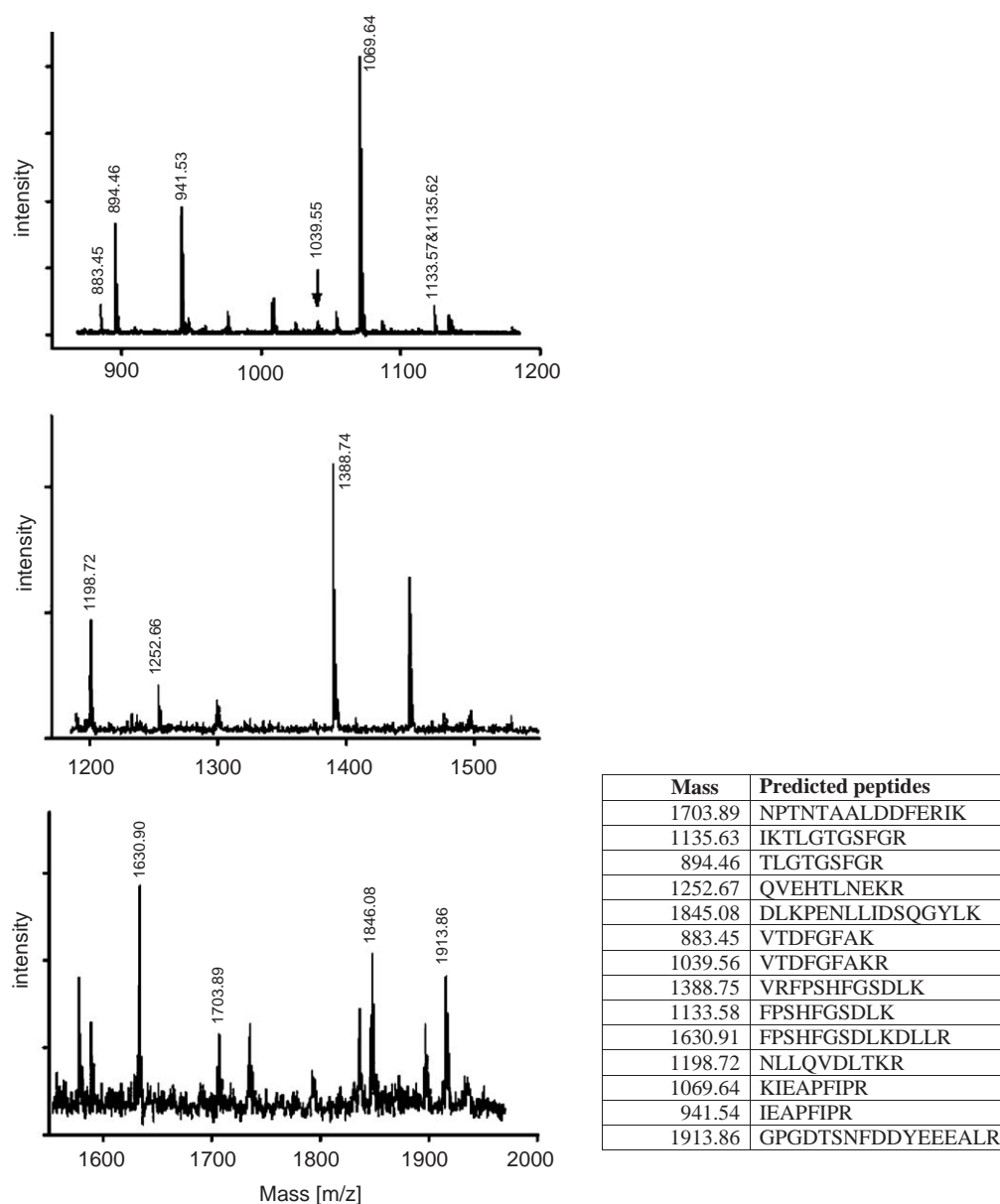


Fig. 3. MALDI-TOF analysis of purified kinase after cleavage by trypsin. Table shows the predicted sequence of the peptides that matched to several regions along the sequence of *Drosophila melanogaster* PKA, C31751.

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1  MGNNATTSNKKVDAAETVKEFLEQAKEEFEDKWRRNPTNTAALDDFERIKTLTGSFGRV
61  MIVQHKPTKDYYAMKILDKQKVVKLKQVEHTLNNEKRILQAIQFPFLVSLRYHFKDNSNLY
121  MVLEYVPGGEMFSHLRKVGRFSEPHSRFYAAQIVLAFEYLHYLDLIYRDLKPENLLIDSQ
181  GYLKVTDFGFAKRVKGRTWTLCGTPEYLAPEIILSKGYNKAVDWWALGVLVYEMAAGYPP
241  FFADQPIQIYEKIVSGKVRFPSHFGSDLKDLLRNLLQVDLTKRYGNLKAGVNDIKNQKWF
301  ASTDWIAIFQKKIEAPFIPRCKGPGDTSNFDDYEEALRISSTEKCAKEFAEF

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Fig. 4. Amino acid sequence of cAMP-dependent catalytic chain of *Drosophila melanogaster* (C31751). The matched peptides from *M. sexta* are shown underlined.

human catalytic subunit of PKA. This similarity is in good agreement with the conclusion of other authors that the catalytic subunit of PKA is highly conserved from lower to higher eukaryotes (Foster et al., 1984; Taylor et al., 1990; Denis et al., 1991; Haq et al., 2000; Pfister and Storey, 2002).

3.2. Characterization of *M. sexta* PKA catalytic subunit

The ability of purified enzyme to phosphorylate Kemptide was investigated. This is a synthetic heptapeptide corresponding to a part of the phosphorylation site of porcine liver pyruvate kinase that is an excellent substrate for PKA (Kemp et al., 1977; Foster et al., 1984; Denis et al., 1991; Haq et al., 2000; Pfister and Storey, 2002). The enzyme showed a high affinity for Kemptide (Fig. 5). The apparent K_m value obtained with this peptide was $31 \mu\text{M}$ and K_m value for Mg-ATP was $39 \mu\text{M}$. These values are within the range reported for the enzyme from other sources (Denis et al., 1991; Mehrani and Storey, 1995) including the goldenrod gall insects (Pfister and Storey, 2002).

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

Histones serve as substrates for many serine/threonine kinases and can be utilized as *in vitro* PKA substrate (Foster et al., 1984; Haq et al., 2000). We used histones as phosphate acceptor to monitor the purification. However, histones are a much poorer substrate for the

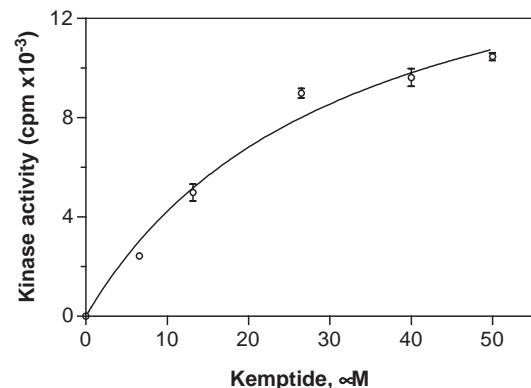


Fig. 5. Concentration curve for Kemptide of Fat body PKA catalytic subunit activity. PKAc activity was measured in the presence of various concentrations of Kemptide, 0.2 mM ATP and 0.5 mM magnesium acetate. Assays were performed at pH 7. K_m value is $31 \pm 7 \mu\text{M}$.

insect kinase than Kemptide, and the K_m value for histones was $0.73 \pm 0.01 \text{ mM}$.

3.3. PKA inhibitors

The effect of different kinase inhibitors on the ability of the purified enzyme to phosphorylate Kemptide was investigated (Fig. 6). The strongest inhibition was found with the synthetic peptide corresponding to residues 5–24 of mammalian inhibitor protein (PKI) that is a potent and specific inhibitor of PKA (Cheng et al., 1986). Approximately 90% of the enzyme activity was inhibited in the presence of 70 nM PKI 5–24 whereas complete inhibition was reached at 700 nM (Fig. 5). The fact that a specific inhibitor of PKA produced complete inhibition of the kinase activity of purified PKA indicated that the presence of contaminating kinases is highly unlikely. H-89 is also a very potent inhibitor of PKA (K_i 48 nM) and we also found high inhibitory effect on the *M. sexta* enzyme being 70% of the activity inhibited by 700 nM . This effect is in the same range previously reported for other PKA kinases (Chijiwa et al., 1990).

Staurosporine aglycon, which is a very potent PKC inhibitor (Fabre et al., 1993), partially inhibited PKA at 50 nM and no further inhibition was observed even at micromolar range ($1.5 \mu\text{M}$). HA1077, a selective calcium/calmodulin protein kinase inhibitor (Takizawa

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

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

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

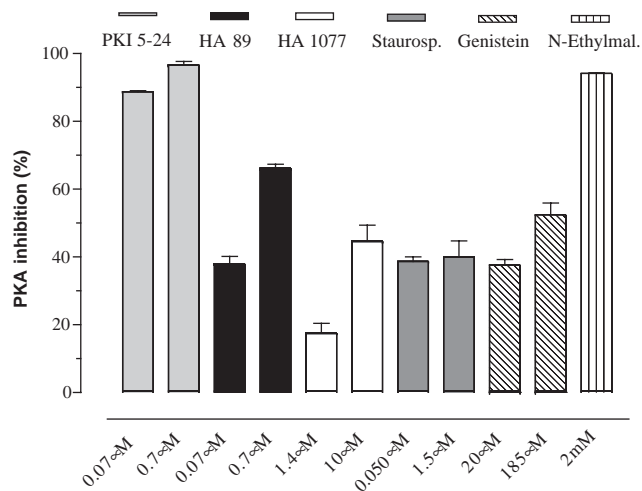


Fig. 6. Effect of inhibitors on the activity of catalytic subunit of *M. sexta* fat body PKA. The percentage of inhibition was calculated using the activity in the absence of inhibitor. Results represent the mean ± SEM (*n* = 3).

et al., 1993) and genistein, a potent tyrosine kinase inhibitor (Akiyama et al., 1987) also showed a partial inhibitory effect only in micromolar range. Inhibitory effect of non-specific inhibitors at higher concentration is a common observation among kinases, as they are highly conserved and recognize very similar substrate sequence (Taylor et al., 1990). We also examined the role of thiol groups in the enzyme activity by preincubating the enzyme with different concentrations of ethylmaleimide, a sulfhydryl group blocker. The activity decreased at increasing concentration of ethylmaleimide and complete inhibition was obtained at 2 mM. This result confirms that the catalytic activity of *M. sexta* PKA requires sulfhydryl groups as previously shown for PKA from other sources (Taylor et al., 1990; Haq et al., 2000).

3.4. Substrate specificity

In general kinases are classified into two groups, serine/threonine and tyrosine kinases. PKA catalyzes the transfer of the γ -phosphate from ATP to serine/threonine residues of substrate proteins (Taylor et al., 1990). We examined the amino acid specificity of the

purified enzyme by comparing the ability of the enzyme phosphorylating different synthetic peptides containing serine, threonine or tyrosine as phosphate acceptor residue. These peptides have sequences that are specifically recognized by different types of kinases and also they are compatible with the simple assay in which binding to phosphocellulose is used to separate ³²P-labeled peptide from ³²P-ATP and ³²Pi. The threonine-containing peptide is a protein kinase C substrate derived from epidermal growth factor receptor (Heasley and Johnson, 1989) whereas tyrosine-containing peptide derived from pp60^{src} protein of Rous sarcoma virus (Casnellie et al., 1982). As shown in Table 3, *M. sexta* fat body PKA only recognized serine residues as phosphate acceptor, neither threonine nor tyrosine containing peptides were phosphorylated. Like mammalian PKA, which recognizes a serine in the physiologically relevant protein substrates (Hjelmquist et al., 1974; Strålfors and Belfrage, 1983), the insect enzyme is also a serine kinase. We further examined substrate sequence specificity of the purified enzyme using synthetic peptides containing a serine residue flanked by different amino acids. The PKA recognition motif has been identified to be Arg-Arg-X-Ser- and this sequence is present in the Kemptide substrate. Two basic residues, usually arginine dyad, separated by a single residue from the phosphorylation site have been observed to be important for primary sequence recognition and substrate binding (Kemp et al., 1977; Feramisco et al., 1980; Denis et al., 1991). Replacement of either of these Arg, even by another basic residue is sufficient to increase *K_m*. Replacement of Arg → Lys at -3 position increased *K_m* by 90 fold, while Arg → Lys at -2 position increased *K_m* by 16 fold of vertebrate kinase (Kemp et al., 1977). A basic residue at -6 position relative to phosphorylation site enhance mammalian enzyme activity, while it had negative effect on the yeast enzyme (Prorok and Lawrence, 1989; Denis et al., 1991). We tested two peptides containing single Ser. One of them has a Thr at -2 instead of Arg. This is Syntide 2 which is a specific substrate for calcium/calmodulin protein kinase (Hashimoto and Soderling, 1987) and proved to be a poorer substrate than Kemptide (Table 3). A similar observation was reported for the *Microsporum gypsum* PKA which

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

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

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

phosphorylates Syntide 2 although at a lower rate than Kemptide (Haq et al., 2000).

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

The physiological substrates of *M. sexta* PKA are unknown. The components of the mechanism of TG mobilization in the insect fat body are potential targets of PKA action given the fact that AKH-induced lipid mobilization involves PKA activation (Arrese et al., 1999). The regulation of TG-lipase activity must be a critical point on the regulation of lipid mobilization. Previously we showed that TG-lipase is a phosphorylatable protein that can be phosphorylated by the bovine catalytic subunit of PKA but the phosphorylation did not affect the lipase activity (Arrese and Wells, 1994). Because the possibility of having non-specific phosphorylations due to the use of bovine PKAc could not be ruled out in that experiment, a definitive conclusion was not possible. The catalytic subunit of PKA from the insect was required in order to verify that observation. For that purpose a preparation of fat body TG-lipase was incubated with purified kinase in the presence of [γ - 32 P]ATP. The preparation was analyzed by SDS-PAGE followed by autoradiography. Fig. 7A shows the SDS-PAGE in which the major band of 76 kDa corresponds to the TG-lipase. Other minor component can also be seen, particularly a protein band

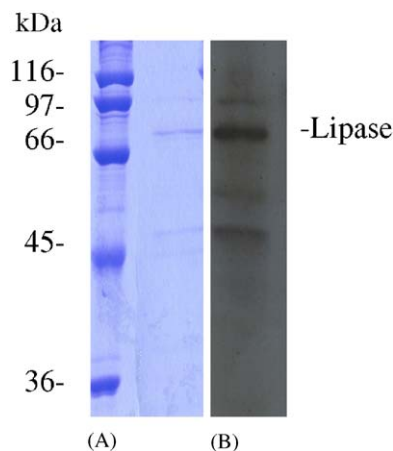


Fig. 7. Phosphorylation of *M. sexta* fat body TG-lipase. Partially purified TG-lipase was incubated with [γ - 32 P]ATP and purified catalytic subunit from *M. sexta* fat body. After incubation proteins were separated on 12% SDS-PAGE. Gel was stained with Coomassie and dried (A), and exposed for autoradiography (B) to visualize 32 P incorporation.

of 50 kDa. This contaminant was present in the TG-lipase preparation. We do not have information on the nature of this protein rather than co-purified with the TG-lipase after several chromatographic steps. It is unknown whether this protein is in some way related to the role of the lipase.

The autoradiography (Fig. 7B) shows that TG-lipase was phosphorylated in vitro by the purified catalytic subunit of PKA suggesting that the lipase could be a physiological substrate of this kinase.

The enzymatic activity of the phosphorylated and unphosphorylated form of the enzyme was compared hydrolyzing [3 H]-triolein as micelles of Triton X-100. For this purpose two types of incubation were performed. On one hand TG-lipase was incubated with purified catalytic subunit of PKA in the presence (phosphorylated) and absence (control) of ATP as indicated in Material and Methods. Afterwards the lipase activity of both types of preparations was measured. We found that the phosphorylated and unphosphorylated (control) form of TG-lipase showed almost the same enzymatic activity hydrolyzing

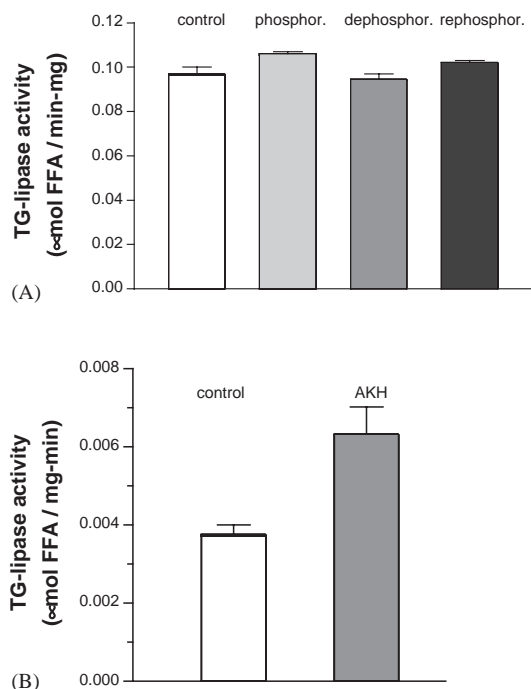


Fig. 8. (A) Effect of phosphorylation on the activity of TG lipase. Partially purified lipase was phosphorylated using ATP in the presence of the catalytic subunit of *M. sexta* PKA. In “Control” samples, the lipase activity was determined using the enzyme as obtained after the purification. In “Phosphorylated” samples, the lipase was phosphorylated by PKAc prior to measure the lipase activity. In “Dephosphorylated” samples, the lipase was dephosphorylated by alkaline phosphatase prior to measure the activity and for “Rephosphorylated” samples, the lipase was incubated with alkaline phosphatase followed by PKAc incubation before to measure the lipase activity. The activity hydrolyzing [3 H]-triolein was determined as indicated in Materials and Methods. Results represent the mean \pm SEM ($n = 3$). The difference of the means is not significant. (B) Effect of AKH on the activity of TG lipase present in fat body homogenates. Results represent the mean \pm SEM ($n = 3$). The difference of the means is significant ($P = 0.026$).

[3 H]-triolein as micelles of Triton X-100 (Fig. 8A). The activity of phosphorylated lipase was slightly higher than the control (10% increase) but this difference was not statistically significant. Panel 8B shows the TG-lipase activity of fat body homogenates from control and AKH treated insects. As previously shown, AKH induced a significant increase—68%—in lipase activity of the homogenates.

The fat body TG-lipase has several properties in common with the hormone-sensitive lipase—HSL—from the vertebrate adipose tissue (Arrese and Wells, 1994). Among those is that HSL is phosphorylated by the catalytic subunit of PKA from adipose tissue. However the in vitro phosphorylation of isolated rat adipose tissue HSL with catalytic subunit of cAMP-dependent protein kinase results in an up to 3-fold increase in the lipase activity against triacylglycerol (Strålfors and Belfrage, 1983; Olsson et al., 1984). Several phosphorylation sites have been disclosed in

HSL. Initially, phosphorylation of Ser 563 by PKA was identified as the regulatory site responsible for the phosphorylation-induced increase in hydrolytic activity. But PKA can also phosphorylate two other serines—659 and 660—and mutational analysis proved that phosphorylation of these two sites are responsible for in vitro activation of HSL. Also these sites are phosphorylated in adipocytes in response to stimulation of lipolysis. The role of phosphorylation of Ser 563 remains elusive. Apart from those three PKA phosphorylation sites, in quiescent cells HSL is phosphorylated in vivo at Ser 565 named the basal site. Other kinases phosphorylate HSL at the basal site and this impairs the phosphorylation of Ser 563 by PKA. However, the finding that Ser 563 is not essential for HSL activation raises some question about the antilipolytic role of Ser 565 phosphorylation (Holm et al., 2000). In any case the in vitro phosphorylation of HSL by PKA increases the hydrolytic activity of this enzyme hydrolyzing TG in an artificial substrate. Unlike the vertebrate lipase, PKA phosphorylation of the insect TG-lipase did not increase its hydrolytic activity. By analogy with HSL the insect lipase could have phosphorylated sites that impair the phosphorylation of regulatory sites. Even though the TG-lipase was purified from quiescent insects in which the enzyme is expected to be dephosphorylated the presence of previously phosphorylated sites on TG-lipase was examined. For this purpose, TG-lipase was incubated with alkaline phosphatase to promote dephosphorylation, and subsequently it was phosphorylated by incubation with PKAc and ATP. Parallel incubations that were done in the presence of [γ - 32 P] $_{4}$ ATP and analyzed by SDS-PAGE followed by autoradiography showed the phosphorylation of the lipase (data not shown). The comparison of the activity of dephosphorylated and rephosphorylated lipase showed no significant differences (Fig. 8A). Rephosphorylated lipase exhibited a slightly higher activity (8% increase) than the dephosphorylated lipase but this difference is not statistically significant. This result indicates that phosphorylation of TG-lipase does not constitute the main or, at least, the only step required for activation of lipolysis. Other proteins and/or mechanisms of activation must be also involved in the activation of lipolysis in the insect fat body.

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