Primary role of adipokinetic hormone in the formation of low density lipophorin in locusts

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Abstract It was demonstrated that the primary action of adipokinetic hormone (AKH) is to stimulate calcium ion uptake into the fat body cell, subsequently causing the formation of diacylglycerol from triacylglycerol. Furthermore, it was also shown that AKH is not directly responsible for increased diacylglycerol uptake by lipophorin from the fat body. The diacylglycerol level of the fat body was found to increase by an average of 2.4-fold after 90 min of incubation in the presence of AKH. Calcium ion was also found to be essential in the action of AKH on the fat body. Supporting this is the observation that calcium ionophore mimics the AKH action in vivo and in vitro; injection of calcium ionophore into adult locusts as well as incubation of hemolymph with fat body and ionophore caused the transformation of high density lipophorin to low density lipophorin. When the fat body, preincubated with or without AKH, was reincubated with hemolymph, diacylglycerol uptake by lipophorin occurred for both incubations. In some sets of experiments, low density lipophorin particles were formed even in the hemolymph that was incubated with fat body preincubated without AKH, indicating that AKH is not directly responsible for its formation. Calcium ion was found not to be necessary for the diacylglycerol uptake process to occur. —**Lum, P. Y., and H. Chino.** Primary role of adipokinetic hormone in the formation of low density lipophorin in locusts. *J. Lipid Res.* 1990. **31:** 2039–2044.

Supplementary key words fat body • triacylglycerol • diacylglycerol • diacylglycerol uptake • lipolysis • calcium ion • calcium ionophore

Lipophorin (1), a major lipoprotein in the hemolymph of most insects, is a reusable shuttle that transports various lipids between tissues (2, 3). One of the most significant lipids is diacylglycerol, whose transport from the fat body is greatly stimulated during flight (4). Injection of insect adipokinetic hormone (AKH) into locust and tobacco hornworm induces lipophorin (high density lipophorin, HDLp) to load large amounts of diacylglycerol to form a particle lower in density, called low density lipophorin (LDLp) (4–7). The formation of the LDLp involves the association of a low molecular weight apoprotein, apolipophorin-III (apoLp-III, mol wt 17,000–20,000) with the lipophorin particle (8–10). Recently, the complete transformation of HDLp to LDLp was demonstrated in vitro by incubating purified lipophorin and apoLp-III with isolated fat body in the presence of AKH (11). Partial transformation of HDLp to LDLp in vitro was also demonstrated by other research groups (12, 13). However, the primary action site of AKH has not been totally elucidated, although in the previous paper we proposed that AKH stimulates lipolysis in the fat body, subsequently converting triacylglycerol to diacylglycerol by primarily stimulating calcium ion uptake (11). We feel it is necessary to present more substantial evidence concerning the primary role of AKH in the mechanism of LDLp formation since many experiments, in vivo and in vitro, involve the action of AKH (4–7, 11–13). In this report, we address the question of the primary site of action of AKH, the mode of the AKH action, and its connection to the increased uptake of diacylglycerol by lipophorin. The role of calcium ions in the action of AKH and the loading of diacylglycerol by lipophorin was also tested in vitro and evidence is presented concerning the crucial role of calcium ion in the primary action of AKH.

MATERIALS AND METHODS

Animals and chemicals

Locusts (*Locusta migratoria*) were reared under crowded conditions on orchard and pampas grass and bran at 30°C with continuous illumination. Adult male locusts, 3–6 weeks after final molt, were used in all experiments except in the in vivo experiments, in which male adults, 3–4 weeks after final molt, were used. Hemolymph was collected according to the flushing-out method (14). Locust adipokinetic hormone (15) was purchased from Peninsula Laboratories (San Carlos, CA). Monoacylglycerol (monostearin), internal standard in lipid analy-

Abbreviations: AKH, adipokinetic hormone; HDLp, high density lipophorin; LDLp, low density lipophorin; apoLp-III, apolipophorin III; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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sis, and calcium ionophore A23187 were obtained from Sigma (St. Louis, MO). Protein standards for SDS-PAGE were purchased from Bio-Rad Laboratories (Richmond, CA). Double-glass-redistilled water was used throughout.

Lipid extraction and analysis

Lipids were extracted from the fat body or hemolymph with chloroform–methanol 2:1. The same solvent system was used to extract lipids from isolated lipophorin. Before extraction, a known amount of monostearin was added to the samples as internal standard. Diacylglycerol content was analyzed using the Iatroscan (model Th-10) essentially according to Ackman (16) as described by Chino, Kiyomoto, and Takahashi (11). The solvent system used in the rod chromatography is benzene–methanol 60:1.5.

Protein assay

Protein was assayed according to the method of Lowry et al. (17), with purified delipidated HDLp as standard.

Gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 10% gel slab according to Laemmli (18).

Density gradient ultracentrifugation

Hemolymph was subjected to KBr density gradient ultracentrifugation essentially according to Shapiro, Keim, and Law (19) as described by Chino, Downer, and Takahashi (7). Centrifugation time was 4 h.

Dissection of fat body

The fat body, distributed longitudinally along both sides of the midgut, was dissected and divided to give four parts, which were then distributed into four small beakers. It should be noted that the fat body varies from one specimen to another. The fat body was dissected with the tracheal system still attached, to minimize any damage to the fat body tissue during dissection. Fat body from different specimens was pooled into beakers by distributing the four sections from each specimen so that each beaker would contain essentially the same kind and approximately the same amount of fat body. The amount of fat body used in different sets of experiments varied from 80 to 100 mg/beaker. This distribution method is very important in our experiments as it serves to minimize experimental variations which are so often caused when using different locust specimens. This method limits the maximum number of incubations to four in one set of experiment. Dissected fat body was washed with about 50 ml of insect Ringer (120 mM NaCl, 15 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, and 5 mM PIPES buffer, pH 7.0), blotted, and weighed before incubation.

Incubation of fat body

Incubations of fat body with and without AKH were carried out in small beakers containing 1.1 ml insect Ringer in a humid, oxygen-saturated chamber placed in a gently shaking water bath (30°C) essentially according to Chino et al. (11). The final concentration of AKH used was 180 nM. After incubation, the fat body was blotted and homogenized in chloroform–methanol 2:1 to extract lipids. The amount of diacylglycerol (peak area of diacylglycerol/peak area of monoglycerol per mg wet weight fat body) was determined using the Iatroscan. Spontaneous leaking of lipids during incubation was checked by assaying aliquots of 50 μl at time intervals. The level of diacylglycerol in fat body was expressed by taking the diacylglycerol amount at 0 min incubation time to be a level of one.

To test the role of Ca^{2+} in the action of AKH, fat body was washed several times in a total of about 500 ml of Ca^{2+}-free insect Ringer before incubation with AKH in a Ca^{2+}-free incubation medium with EGTA added to a final concentration of 20 mM. We found that the addition of EGTA to the incubation medium did not cause the disintegration of the fat body as no artificial leaking was detected during incubation. This is probably because of the improved dissection method of the fat body. Incubation in the presence of calcium ion was carried out as above.

Assay of diacylglycerol uptake by hemolymph lipophorins from fat body preincubated with and without AKH

One ml of hemolymph (3–4 mg protein/ml), dialyzed several times against a total of 2 liters of insect Ringer, was used in each incubation. The fat body used was preincubated either with or without AKH. After preincubation, the fat body was washed thoroughly with insect Ringer before reincubating with hemolymph. Incubation conditions were essentially the same as those mentioned for the incubation of the fat body. Fifty-μl aliquots were taken from the incubation medium at 0, 90, and 180 min incubation time. Lipids were then extracted from each aliquot with chloroform–methanol 2:1 and subjected to analysis with the Iatroscan. The amount of diacylglycerol for each aliquot was calculated as follows: peak area of diacylglycerol/peak area of monoacylglycerol per 50 μl hemolymph. The level of diacylglycerol in hemolymph is expressed by taking the diacylglycerol amount at 0 min incubation time to be a level of one. Leakage was also checked by incubating preincubated fat body with insect Ringer instead of hemolymph.

To test the necessity of Ca^{2+} in diacylglycerol uptake, Ca^{2+}-free incubation medium was obtained by dialyzing hemolymph against Ca^{2+}-free insect Ringer and adding EGTA to a final concentration of 20 mM to the incuba-

tion medium just before incubation. The preincubated fat body to be used in Ca^{2+}-free incubations was rinsed several times with Ca^{2+}-free insect Ringer before incubations. Other procedures were as above.

In vivo injection of calcium ionophore

Calcium ionophore A23187 (1 mg/ml) dissolved in dimethylformamide–ethanol 3:1 (v/v) was injected into locusts (5 μl/insect). As controls, other locusts were also injected with AKH (5 μl of 2 μM AKH/insect), dimethylformamide–ethanol 3:1 (5 μl/insect), and insect Ringer (5 μl/insect). For each type of injection, three locusts were used. Ninety min later, hemolymph was collected using the flushing-out method and subjected to density gradient ultracentrifugation. Yellow LDLp and HDLp bands were collected with a Pasteur pipet and subjected to lipid analysis, protein assay, and SDS-PAGE.

RESULTS AND DISCUSSION

Mode of action of AKH

Effect of AKH on the diacylglycerol level of the fat body. Until now, the in vivo or in vitro effect of AKH was judged by analyzing the increase in the level of diacylglycerol in hemolymph or in isolated lipophorin (4–7, 11–13). Although it has been speculated that AKH primarily acts on the fat body, it is necessary to make clear its primary action site and whether or not its presence is necessary for the increased loading of diacylglycerol from the fat body. When the fat body is incubated with AKH in insect Ringer, in other words, without the transport shuttle lipophorin, the mode of action of AKH on the fat body can be observed.

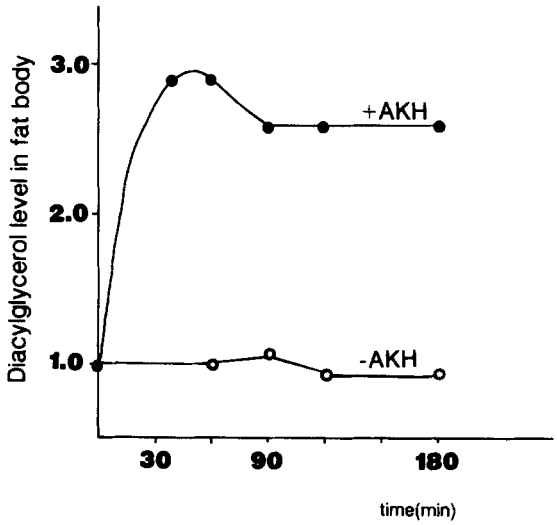

Fig. 1. Time course of diacylglycerol level of the fat body in the presence (solid circle) or absence (open circle) of AKH. Fat body was incubated in insect Ringer in the presence or absence of AKH (see Methods for details) and the amount of diacylglycerol was determined. The level of diacylglycerol was calculated by taking the diacylglycerol amount at 0 min to be a level of one.

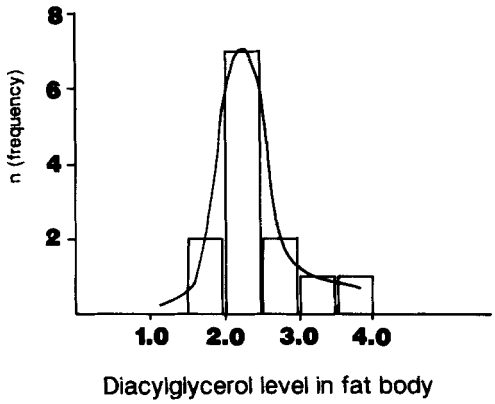

Fig. 2. Histogram of diacylglycerol levels of the fat body after 90 min incubation with AKH. For each set of experiments, the diacylglycerol amount of fat body at 0 min incubation time was determined and taken to be a level of one.

Time course experiments on the effect of AKH on diacylglycerol level in the fat body showed that response to AKH was rapid. Between 30 and 60 min of the incubation time, the level of diacylglycerol was at its highest. At 90 min, the level was slightly lower and the graph showed a plateau after that point (**Fig. 1**). On the other hand, practically no change was observed in the diacylglycerol level throughout the incubation when the fat body was incubated without AKH (Fig. 1). Although it was found that the absolute diacylglycerol amount differs with different sets of experiments due to different sets of fat body, the shape of the graph remained the same. In 12 sets of experiments, the initial absolute amount of diacylglycerol (at 0 min) per 100 mg wet weight fat body was 320 μg $\pm$ 120 (SD). The peak between 30 and 60 min incubation time is most likely due to diacylglycerol being formed in excess at first, and subsequently, reduced slightly to give a plateau due to a feedback mechanism because lipophorin, the diacylglycerol acceptor, was not present in the incubation medium. In other words, the level of diacylglycerol in the fat body can be said to be saturable. In order to check the saturation level of diacylglycerol in the fat body, its level after 90 min of incubation was determined. The diacylglycerol level of the fat body increased an average of 2.4-fold with the treatment of AKH whereas no change was observed in the diacylglycerol level of the fat body treated without AKH at 90 min incubation time. **Fig. 2** shows a histogram of the values of diacylglycerol level in the fat body observed after 90 min of incubation with AKH for different sets of experiments. It can be seen that the most common diacylglycerol level at 90 min was between 2.0 and 2.4. Spontaneous leaking of diacylglycerol was not detected during incubation.

Role of Ca^{2+} in the AKH action. Incubation of fat body in the presence of AKH without Ca^{2+} did not result in any

significant increase in the diacylglycerol content (**Fig. 3**). This indicates that extracellular calcium ion is necessary for AKH to exert its effect on the fat body. In fact, calcium ionophore was found to be a perfect mimic of AKH in vivo. As illustrated in **Fig. 4A,** LDLp particles were formed in the hemolymph of locusts injected with ionophore whereas they were not observed in hemolymph of locusts injected with insect Ringer or the calcium ionophore solvent, dimethylformamide–ethanol solution. SDS-PAGE showed that apoLp-III was also associated with the LDLp particles formed as a result of ionophore action just as in the AKH-induced LDLp particles (**Fig. 5**). Lipid analysis showed that the amount of diacylglycerol for both the AKH-induced and the ionophore-induced LDLp particles was almost the same (**Table 1**). The ionophore effect was also demonstrated in vitro (Fig. 4B). When fat body was incubated with hemolymph and ionophore, the formation of LDLp was observed. Although the LDLp band is rather broad, as seen from Fig. 4B, such a broad band was also often observed for in vitro AKH incubations (11). Since calcium ionophore is known to transport calcium ion across the plasma membrane, we conclude that the primary action of AKH is to stimulate the uptake of calcium ion into the fat body. Thus, we can assume that the fat body lipase is activated via a Ca^{2+}-dependent protein kinase and/or cyclic AMP (20, 21) resulting in an increase of diacylglycerol in the fat body. Another research group (21) found that calcium ionophore increased the diacylglycerol level in locust fat body in vitro. We have reconfirmed this (data not shown). This activation of lipolysis in the fat body by insect AKH seems essentially analogous to the activation of lipolysis by lipolytic hormones in mammalian adipose tissue (22).

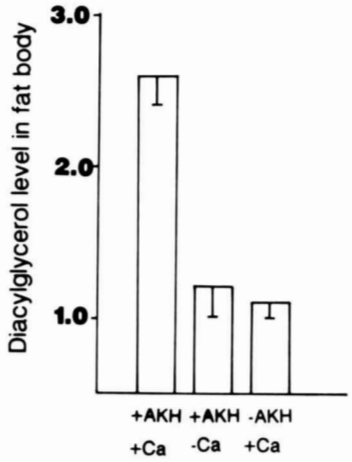

Fig. 3. Effect of Ca^{2+} on the action of AKH. Fat body was incubated with AKH or without AKH in the presence of Ca^{2+} or with AKH in the absence of Ca^{2+}. After 90 min of incubation, the diacylglycerol level of the fat body was determined. Other explanations as in Fig. 2. Each histogram represents the mean of four determinations with ± SD (vertical lines).

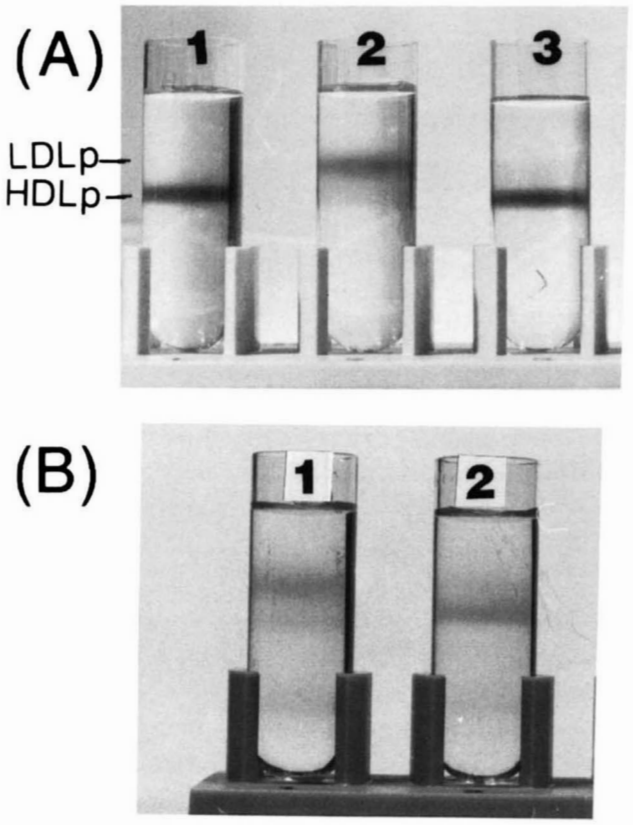

Fig. 4. A: KBr density gradient ultracentrifugation of hemolymph of locusts injected with calcium ionophore. Injection of insect Ringer as control (1), calcium ionophore A23187 (2), and dimethylformamide–ethanol (solvent) as control (3). The original color of the lipophorin band is deep yellow due to carotenoid pigments. The LDLp band observed in the ultracentrifuged hemolymph of locusts injected with ionophore had the same density (1.065 g/ml) as that of the LDLp band of AKH-injected locusts (not shown here). B: KBr density gradient ultracentrifugation of hemolymph incubated with fat body and 20 μg ionophore (1) or solvent as control (2).

Diacylglycerol uptake by hemolymph lipophorins from fat body preincubated with or without AKH

By first increasing the diacylglycerol level of the fat body with AKH and then incubating the fat body with hemolymph, we should be able to determine whether the presence of AKH is necessary for the increased uptake of diacylglycerol. As shown in **Fig. 6,** it was found that diacylglycerol uptake occurred whether the hemolymph was incubated with fat body preincubated with or without AKH. This observation is supported by the result illustrated in Fig. 4B, 2; the HDLp band became broader and its density became slightly lower even in the absence of ionophore (control experiment). The difference between the diacylglycerol level in the hemolymph of the two conditions (with or without AKH) after incubation reflects the difference in the amount of diacylglycerol that is available in the fat body. Lipid analysis also showed that the

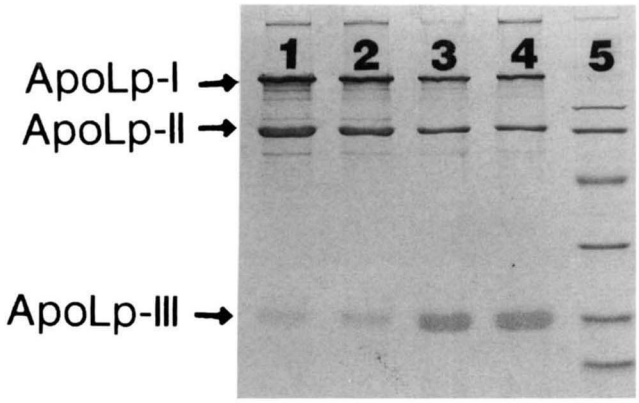

Fig. 5. SDS-PAGE of lipophorins isolated by density gradient ultracentrifugation. Each lane has 8–9 μg protein. Lane 1, HDLp of Ringer-injected locusts; lane 2, HDLp of dimethylformamide–ethanol-injected locusts; lane 3, LDLp of AKH-injected locusts; lane 4, LDLp of ionophore-injected locusts; lane 5, standard proteins with known molecular weights, from top to bottom; phosphorylase b (M_r 92.5 kDa), bovine serum albumin (M_r 66.2 kDa), bird egg ovalbumin (M_r 45 kDa), carbonic anhydrase (M_r 31 kDa), soybean trypsin inhibitor (M_r 21.5 kDa), and lysozyme (M_r 14.4 kDa).

diacylglycerol levels of the fat body decreased significantly after incubation with hemolymph. A typical example showed that the diacylglycerol level of a fat body, whose level was 2.0 after preincubation with AKH, was reduced to 1.0 after incubation with hemolymph (see Methods for the expression of diacylglycerol level). The diacylglycerol level of a fat body preincubated without AKH (thus whose diacylglycerol level was 1.0) became 0.5 after reincubation with hemolymph. Calculations also showed that about 70% or more of the diacylglycerol depleted from the fat body was found in the hemolymph after 3 h of incubation. From these results of in vitro experiments we conclude that lipophorin will continue to load diacylglycerol as long as there is diacylglycerol available in the fat body or until its own diacylglycerol level reaches a maximum even in the absence of AKH. Indeed, in some sets of experiments, a distinct LDLp band was formed even when the hemolymph was incubated with fat body

TABLE 1. Typical data on the diacylglycerol content of lipophorin after in vivo injection of locusts with calcium ionophore, AKH, dimethylformamide–ethanol, or insect Ringer

In vivo Injection of Locusts	Diacylglycerol Content
	μg/100 μg protein
Calcium ionophore	152
AKH	165
Dimethylformamide–ethanol (3:1)	30.3
Insect Ringer	32.1

Ninety min after injection, lipophorin was isolated by density gradient ultracentrifugation and the absolute amount of diacylglycerol was determined by Iatroscan.

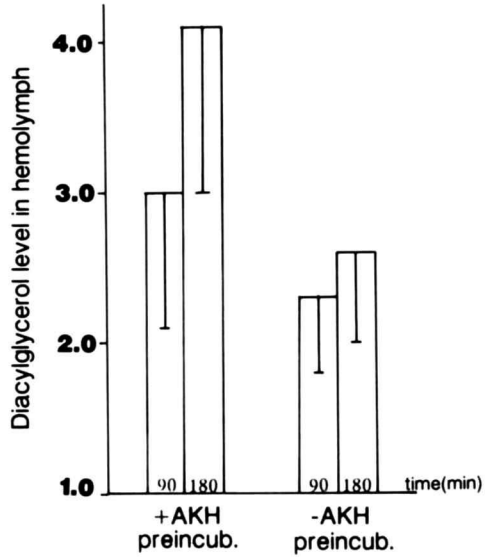

Fig. 6. Diacylglycerol uptake by hemolymph lipophorin. The fat body was preinicubated with (left histograms) or without (right histograms) AKH. After preincubation, the fat body was thoroughly rinsed with Ringer solution to remove AKH. The preincubated fat body was then reincubated with hemolymph (note that no AKH was present in the reincubation medium). Aliquots of 50 μl were removed from the incubation medium at 0 min, 90 min, and 180 min incubation time and the amount of diacylglycerol of each was determined. Diacylglycerol level was calculated by taking the diacylglycerol amount at 0 min to be a level of one. Each histogram represents the mean of eight replicates ± SD (vertical lines).

that was preincubated without AKH (data not shown). The fact that LDLp particles were formed shows that neither the presence of AKH nor the preincubation with AKH was necessary for the formation of LDLp particles,

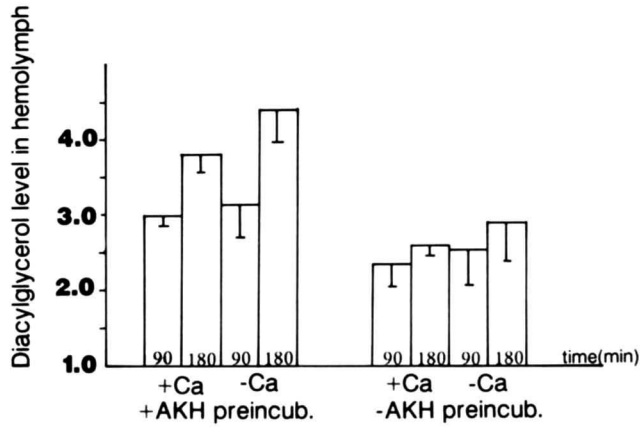

Fig. 7. Effect of Ca^{2+} on diacylglycerol uptake by hemolymph lipophorin. Fat body, preincubated with (left histograms) or without (right histograms) AKH, was reincubated with hemolymph in the presence or absence of Ca^{2+}. Note that Ca^{2+} is always present during preincubation of fat body. It is during the reincubation of fat body (rinsed free of AKH) with hemolymph that the effect of the presence or absence of Ca^{2+} was tested. Aliquots of 50 μl were taken from the incubation medium at 0 min, 90 min, and 180 min incubation time. The diacylglycerol level was calculated by taking the diacylglycerol amount of hemolymph at 0 min to be a level of one. Each histogram represents the mean of three replicates ± SD (vertical lines).

although the increase in the fat body diacylglycerol level caused by the AKH action no doubt plays an important role in the complete transformation of HDLp to LDLp. Recently, Van Heusden and Law (23) reported that the lipid transfer particle is involved in the process of lipid mobilization from fat body and LDLp formation in *Manduca sexta*.

Calcium ion was found not to be necessary in the loading process of diacylglycerol. Results showed that diacylglycerol uptake was not inhibited at all by the lack of Ca^{2+} (**Fig. 7**). In fact, the level of diacylglycerol in the Ca^{2+}-free medium was slightly higher than that of the medium with Ca^{2+}. No explanation can be offered for this as it was not due to disintegration of the fat body because no spontaneous leaking of diacylglycerol was detected during incubation.

We conclude from our present data that AKH only serves to stimulate calcium ion uptake into the fat body cells, thereby stimulating lipolysis, i.e., the formation of diacylglycerol from triacylglycerol, and has no direct part in the loading process of diacylglycerol by lipophorin from the fat body.

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