Calcium and cAMP are second messengers in the adipokinetic hormone-induced lipolysis of triacylglycerols in *Manduca sexta* fat body

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Abstract **We have previously shown that stereospecific hydrolysis of stored triacylglycerol by a phosphorylatable triacylglycerol-lipase is the pathway for the adipokinetic hormone-stimulated synthesis of *sn*-1, 2-diacylglycerol in insect fat body. The current series of experiments were designed to determine whether cAMP and/or calcium are involved in the signal transduction pathway for adipokinetic hormone in the fat body. After adipokinetic hormone treatment, cAMP-dependent protein kinase activity in the fat body rapidly increased and reached a maximum after 20 min, suggesting that adipokinetic hormone causes an increase in cAMP. Forskolin (0.1 μM), an adenylate cyclase activator, induced up to a 97% increase in the secretion of diacylglycerol from the fat body. 8Br-cAMP (a membrane-permeable analog of cAMP) produced a 40% increase in the hemolymph diacylglycerol content. Treatment with cholera toxin, which also stimulates adenylate cyclase, induced up to a 145% increase in diacylglycerol production. Chelation of extracellular calcium produced up to 70% inhibition of the adipokinetic hormone-dependent mobilization of lipids. Calcium-mobilizing agents, ionomycin and thapsigargin, greatly stimulated DG production by up to 130%. Finally, adipokinetic hormone caused a rapid increase of calcium uptake into the fat body. Our findings indicate that the action of adipokinetic hormone in mobilizing lipids from the insect fat body involves both cAMP and calcium as intracellular messengers.**—Arrese, E. L., M. T. Flowers, J. L. Gazard, and M. A. Wells. **Calcium and cAMP are second messengers in the adipokinetic hormone-induced lipolysis of triacylglycerols in *Manduca sexta* fat body. *J. Lipid Res.* 1999.** 40: **556–564.**

Supplementary key words AKH • cAMP • calcium • fat body • lipid mobilization • diacylglycerol • *Manduca sexta*

The insect fat body, which combines many of the properties and functions of vertebrate liver and adipose tissue, is the principal site for the storage of lipid (1). Triacylglycerols (TGs) represent about 90% of the total fat body lipid (2). In the tobacco hornworm, *Manduca sexta*, which is widely used as a model insect, 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 (3). Afterwards, the stores of TG start to decline as they are used to sustain energy metabolism during the subsequent non-feeding pupal and adult periods (3, 4).

Unlike vertebrates, in which the stored TGs are completely hydrolyzed prior to export of fatty acids into the plasma, in most insects, fatty acids are released from the fat body as *sn*-1, 2-diacylglycerols (DGs). The DGs are loaded into the hemolymph lipoprotein, lipophorin, causing the transformation of high density lipophorin (HDLp) into low density lipophorin (LDLp) (5).

Two factors of cephalic origin have been shown to activate lipolysis in the fat body: adipokinetic hormone (AKH) and octopamine. AKH, a nonapeptide that is released during flight in many insects, greatly stimulates the secretion of DGs from the fat body (6). Octopamine, a monohydroxyphenolic analogue of noradrenaline whose secretion is stimulated by stress, has been shown to stimulate lipid mobilization in the locust (7) and in the house cricket (8). Octopamine also stimulates lipolysis in adult *M. sexta*, but shows a much smaller effect than AKH (E. L. Arrese and M. A. Wells, unpublished results).

In the first step of lipid mobilization, TGs are hydrolyzed by the action of a lipase. Previously, we purified and characterized a TG-lipase from *M. sexta* fat body (9). Several properties of this enzyme were in common with the vertebrate hormone-sensitive lipase (HSL) which catalyzes the rate-limiting step in mobilization of adipose tissue fatty acids. Like HSL, the *M. sexta* fat body TG-lipase is a phosphorylatable enzyme (9).

In adult *M. sexta*, the activation of the fat body TG-lipase precedes the appearance of DG in the hemolymph, suggesting that AKH stimulates DG secretion by activating

Abbreviations: AKH, adipokinetic hormone; TG, triacylglycerol; DG, diacylglycerol; LDLp, low density lipophorin; HDLp, high density lipophorin; DMSO, dimethylsulfoxide; cAMP, adenosine 3′: 5′ cyclic monophosphate; 8Br-cAMP, 8,bromo-cAMP; A-kinase, cAMP-dependent-protein kinase.

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the fat body TG-lipase (10). Recently, we presented evidence that stored TG is the direct precursor of secreted *sn*-1, 2-DG. Therefore, stereospecific hydrolysis of fat body TG is the pathway for the AKH-stimulated synthesis of *sn*-1, 2-DG (11, 12).

The details of the hormonal regulation of the lipolytic process that takes place in the insect fat body remain to be elucidated. Previous studies in the locust fat body indicated that cAMP (13) and/or calcium (13–15) are involved in mediating the action of AKH mobilizing lipids. As part of a study on the mechanism of regulation of lipolysis insects, the present study was designed to determine whether these two messengers are involved in the signal transduction of AKH in *M. sexta* fat body. Our findings indicate that the action of AKH involves both cAMP and calcium as intracellular messengers.

MATERIALS AND METHODS

Animals

Two- or three-day-old male *M. sexta* adults from a colony maintained in the authors' laboratory were used. This colony was established from *M. sexta* eggs obtained from the USDA, State University Station, Fargo, ND. The colony is maintained according to the basic rearing techniques of Bell and Joachim (16) with minor modifications. Adults were kept at 25°C without food. Animals were decapitated 24 h prior to being used. Before use, the decapitated insects were injected with 13 mg of trehalose dissolved in 20 μl of H_2O (10). After an additional 2 h, the insects were used for experiments. In a previous report we showed that decapitated insects injected with trehalose exhibit a low level of lipolysis, as judged by the level of fat body TG-lipase activity and the concentration of LDLp in the hemolymph, and that after AKH injection, these insects showed activation of the lipase and a high level of DG mobilization from the fat body (10).

Chemicals

M. sexta AKH was purchased from Peninsula Laboratories (Belmont, CA). Ionomycin, thapsigargin, cholera toxin, 8-Br-cAMP, forskolin, 1,9-dideoxyforskolin, and Bapta [1,2-bis(*o*-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid tetra(acetoxymethyl)ester] were obtained from Calbiochem (San Francisco, CA). IBMX (3-isobutyl-1-methylxanthine), dibutyryl-cAMP, cAMP, ATP, PKI (cAMP-dependent protein kinase inhibitor), verapamil, nifedipine, and Grace's insect medium were obtained from Sigma (St. Louis, MO). [γ-$^{32}PO_4$]-ATP was purchased from New England Nuclear Corp. (Boston, MA) and $^{45}CaCl_2$ was from ICN Biomedicals, Inc (Irvine, CA). All other chemicals were of analytical grade.

Injections of chemicals

The water-insoluble compounds, forskolin, 1,9-dideoxiforskolin, IBMX, thapsigargin, ionomycin, nifedipine, were dissolved in dimethylsulfoxide (DMSO). Twenty min after injecting 5 μl of the appropriate solution, the hemolymph was collected, as described previously (10) and used for determination of the relative masses of LDLp and HDLp. The equivalent volume of DMSO or water was injected into the control animals. The final concentration of each compound reached in the hemolymph was calculated considering a total hemolymph volume of 265 μl (4) plus the volume of each compound injected. For each condition, hemolymph from at least three insects was pooled and analyzed for LDLp content.

Measurement of LDLp content

As a measure of the hemolymph DG content, the relative masses of LDLp and HDLp were determined (10). Values for LDLp are expressed as percentage of total lipoprotein (LDLp + HDLp). Changes in [LDLp] percentage were calculated as follows: (LDLp − LDLp control) × 100/LDLp control. For each condition, hemolymph from at least three insects was pooled and analyzed for LDLp content. Four different pools were analyzed with each of the chemicals tested at every concentration. Results are the mean ± SEM (n = 4).

Assay of A-kinase activity

Fat body tissue from three insects was dissected, pooled, and homogenized in 50 mm Tris-HCl, pH 7.4, containing 0.25 m sucrose and 0.1% (v/w) 2-mercaptoethanol. The tissue was homogenized at a ratio of 2 ml of solution per fat body using a Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was centrifuged at 20,000 *g* for 20 min. The proteins kinase activity was determined in the infranatant. For the time course study, fat body and hemolymph were taken at various periods of time after the injection of 100 pmol AKH/insect. Fat body homogenates were assayed for A-kinase activity and the LDLp and HDLp content in the hemolymph were determined. Material from three insects was pooled for each time point.

A-kinase activity was measured by the incorporation of $^{32}PO_4$ into histone. An amount of extract containing 150 μg of protein was added to a reaction mixture which contained 50 mm MOPS (pH 7.0), 2.5 mm $MgCl_2$, 30 mg/ml histone, and 0.2 mm [γ-^{32}P] ATP (5×10^4 cpm/nmol). Incubations were done under three separate conditions according to Honnors, Dhillon, and Londos (17): *a*) with no additions; *b*) in the presence of 20 μm cAMP; *c*) in the presence of 0.7 μm PKI, a specific A-kinase inhibitor. The final volume of the mixture was 0.1 ml. After incubation at room temperature for 10 min, the reaction was terminated by adding 5 μl of 12 N HCl. Seventy μl of the reaction mixture was spotted onto a disc of phosphocellulose paper. Filter papers were washed for 20 min in 50 mm NaCl four times. The dried paper discs were analyzed for protein-bound ^{32}P by liquid scintillation counting. A-kinase activity was proportional to the amount of protein added up to 200 μg and with time up to 10 min when 100 μg of protein was used. Corrections were made for non-A-kinase activities. Results are expressed as corrected A-kinase activity ratios, r = [(a − c)/(b − c)]. Results represent mean ± SEM of three experiments.

Measurement of $^{45}Ca^{2+}$ uptake

Unidirectional calcium influx was measured according to the method of Mauger et al. (18) with minor modifications. Fat bodies were washed with incubation medium [1:10 dilution of Grace's insect medium supplemented with (in g/l): sucrose, 25; KCl, 4.0; $MgCl_2 \cdot 6H_2O$, 2.0; $MgSO_4 \cdot 7H_2O$, 3.0; NaH_2PO_4, 1.0; pH 6.5]. Before removing the fat body, the tissue was excised longitudinally into two pieces that were used as test and control, respectively. Each piece was pre-incubated in 1.6 ml of incubation medium for 2 min at room temperature. Afterwards, the medium was removed and the experiment was started by adding 0.8 ml of incubation medium containing $^{45}CaCl_2$ (10 μCi / ml), with and without 0.4 μm AKH, respectively. Incubations were terminated by addition of 2 ml of ice-cold Grace's solution. The mixture was filtered and washed three times with 2 ml of the ice-cold Grace's solution. The radioactivity associated with the tissue on the filter was then counted in a liquid scintillation counter. Incubation periods of 15, 45, 75, 105, and 135 sec were assayed. Blank values for each time point were estimated by incubating the filters in the absence of tissue. Two independent experiments using four different fat bodies per each time point were performed. Results

were expressed as "% of basal $^{45}Ca^{2+}$ uptake", which was estimated as (cpm test − cpm blank) × 100/(cpm control − cpm blank). Given that an independent experiment gave similar results, the experimental data were combined to obtain the mean and SEM values.

Other procedures

Protein concentration was determined by the Bradford dye-binding assay using bovine serum albumin as a standard (19).

Statistics

Results are presented as the mean ± SEM. Statistical comparisons were made by the Student's *t*-test; $P \leq 0.05$ was considered to be significant.

RESULTS

Time course of A-kinase activation by AKH

To evaluate the messenger role of cAMP, the time course of changes in A-kinase activity induced by the injection of AKH was determined (**Fig. 1**). To ensure maximal response to the hormone, 100 pmol of AKH was injected into each insect. Previous work showed that this dose induces the maximum response 30 min after the injection (10).

Homogenates from non-stimulated fat bodies show A-kinase activity ratios of 0.049 ± 0.011. When insects were treated with AKH, the A-kinase activity ratio rapidly increased (Fig. 1). A 4-fold increase that turned statistically significant ($P = 0.023$) 5 min after the injection was observed. Afterwards, the A-kinase activity ratio persisted 3- to 4-fold above basal level ($P \leq 0.047$). The differences in A-kinase activity ratio observed between 5 to 60 min were not statistically significant. As shown in Fig. 1, the onset of the lipid mobilization measured by the increase in the concentration of hemolymph LDLp began between 5 and 10 min after the injection of AKH. Moreover, the A-kinase activation also preceded the TG-lipase activation in which maximal values were found at ca. 10 min (Fig. 1, inset). To allow an easy comparison, the results of lipase activation previously published (10) are included in the figure as an inset. According to Honnors et al. (17), the ratio between the A-kinase activity in the absence and in the presence of an excess of exogenous cAMP represents an indirect measurement of the cellular cAMP concentration. Because histones are a non-specific substrate for A-kinase, the activities were corrected for non-A-kinase reactions by measuring the activity in the presence of the specific A-kinase inhibitor PKI. Therefore, under these conditions the A-kinase activity ratio is a measure of the intracellular concentration of cAMP.

Effect of agents that raise intracellular cAMP on lipolysis

To further verify the role of cAMP in the lipolytic response, fat bodies were treated either with the diterpene forskolin, which is known to increase intracellular cAMP by directly activating adenylate cyclase (20), or with the membrane-permeable cAMP analogue, 8Br-cAMP. These compounds were injected in conjunction with 150 μm IBMX, which is a phosphodiesterase inhibitor (21). When IBMX was given alone, no modification in the level of LDLp was observed (data not shown).

Concentrations as low as 0.1 μm forskolin produced a

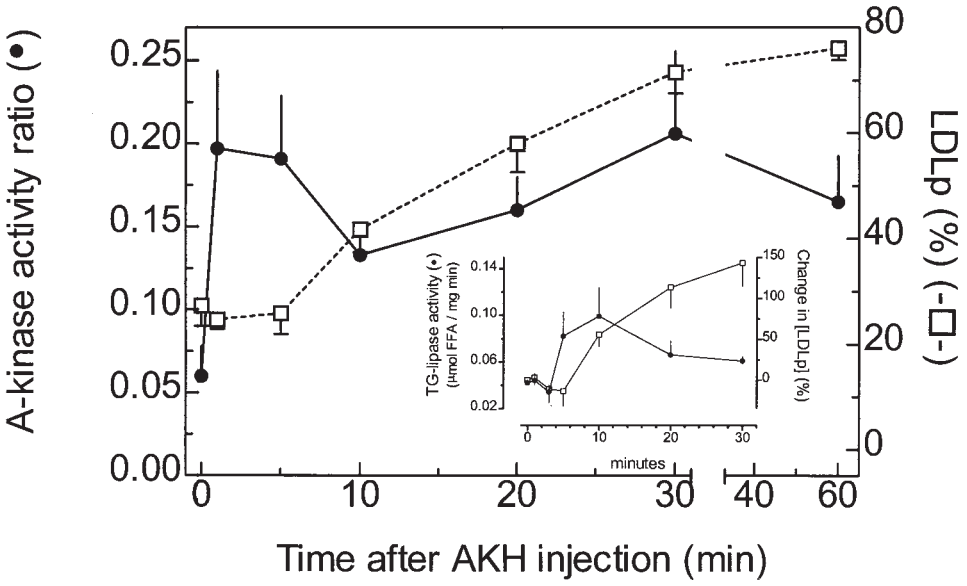

Fig. 1. Time course of the effect of AKH on A-kinase activity ratio and lipid mobilization. Fat body and hemolymph were taken at various periods of time after the injection of 100 pmol AKH/insect. Fat body homogenates were assayed for A-kinase activity and the LDLp and HDLp content in the hemolymph were determined. Material from three insects was pooled for each time point. Results are expressed as corrected A-kinase activity ratios (see Methods) whereas values for LDLp are expressed as percentage of total lipoprotein (LDLp + HDLp). Data represent mean ± SEM of three experiments (n = 3). The inset shows the time course of the TG-lipase activation and the change of LDLp induced by AKH that were previously published (10).

significant ($P = 0.038$) activation of the synthesis of DG, whereas the inactive analog of forskolin, 1,9-dideoxyforskolin, failed to stimulate lipid mobilization (**Fig. 2A**). Up to a 97% increase in the lipolytic response was achieved in the presence of 0.1 μm forskolin. Moreover, a stimulatory effect was also observed in the case of 8Br-cAMP (Fig. 2B). However, at low concentrations of the cAMP analogue, the increases of LDLp content were not statistically significant. Concentrations as high as 150 μm 8Br-cAMP were required to produce a significant change (40%, $P = 0.041$) in the LDLp content. Another cell-permeable cAMP analog, dibutyryl-cAMP, was even less effective than 8Br-cAMP mobilizing DG into the hemolymph (data not shown).

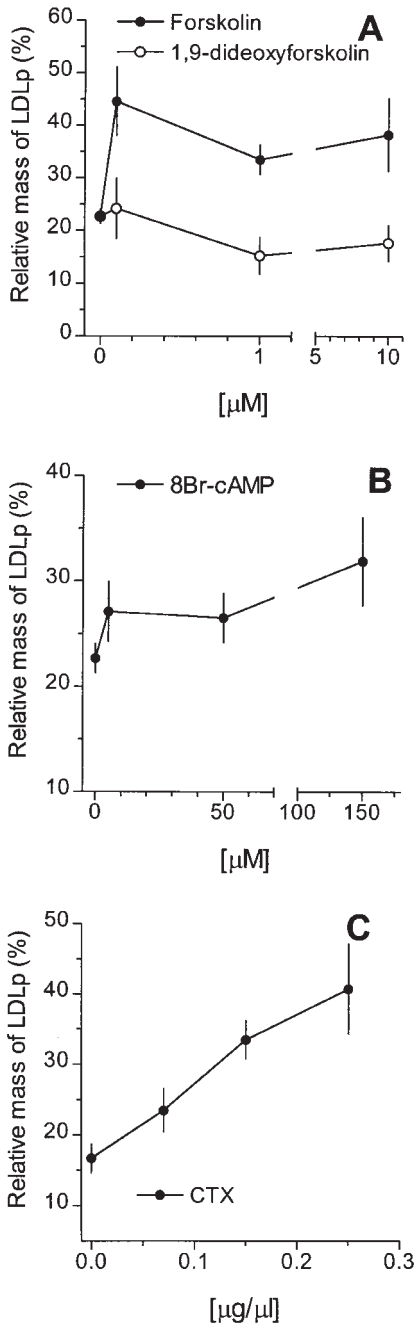

Fig. 2. Effect of agents that raise intracellular cAMP on DG production. Insects were injected with varying concentrations of the following chemicals: A) forskolin and 1,9-dideoxyforskolin, respectively; B) 8Br-cAMP; and C) cholera toxin. After 20 min, hemolymph was collected, pooled and analyzed for LDLp content. The final concentration of each compound reached in the hemolymph was calculated considering a total hemolymph volume of 265 μl (4) plus the volume of each compound injected. For each condition, hemolymph from at least three insects was pooled and analyzed for LDLp content. Four pools were analyzed with each of the chemical tested at every concentration. Results are the mean ± SEM (n = 4).

Further evidence that implicates cAMP as a relevant message for increased lipolysis was obtained by using cholera toxin, which stimulates adenylate cyclase by permanently activating a G_s protein (22). Fig. 2C shows the concentration–response curve for the activation of lipolysis induced by cholera toxin. A marked increase, up to 145% ($P = 0.024$), in lipid mobilization was observed at the maximal concentration of cholera toxin that was assayed.

Effect of a calcium chelator and calcium channel antagonists on the adipokinetic response

To examine the possible involvement of calcium in the activation of lipolysis by AKH, we studied the effect of extracellular calcium chelation on the AKH action. Decapitated insects pretreated with various concentrations of the calcium-chelating agent Bapta were stimulated with AKH (**Fig. 3A**). Increasing concentrations of Bapta in the hemolymph produced an increased inhibition of the response to AKH. Inhibitions of 45% ($P = 0.003$) and 70% ($P < 0.001$) were reached at Bapta concentrations of 10 mm and 20 mm, respectively. In order to investigate whether Bapta affected the viability of the insects, insects were treated with 20 mm Bapta for 10 min followed by 80 mm $CaCl_2$ and AKH. The magnitude of the adipokinetic response of these insects was the same as that of control insects. Therefore, this result proved that the effect of Bapta on AKH action was due to the chelation of calcium rather than an effect on the viability of the insects.

The effect of two different calcium channel blockers, a 1,4-dihydropyridine-type, nifedipine, and the phenylalkylamine, verapamil, on the adipokinetic action was also investigated. As shown in Fig. 3B, at the highest concentration tested, lipolysis was moderately but significantly inhibited in both cases. However, nifedipine proved to be a better antagonist than verapamil. Up to a 30% ($P = 0.038$) inhibition in the secretion of DG was achieved in insects that were injected with 1.0 mm of nifedipine compared to 15% ($P = 0.016$) in the case of the maximal concentration of verapamil.

Effect of Ca^{2+} mobilizing agents on lipolysis

To further explore the role of calcium in the stimulation of lipid mobilization, the effect of two different agents that increase intracellular calcium concentration were studied. The sesquiterpene thapsigargin raises intracellular calcium by inhibiting the Ca^{2+}-ATPase pump of the endoplasmic recticulum without affecting the action of the plasma membrane ATPase (23) or the levels of inositol phosphates. The calcium ionophore ionomycin re-

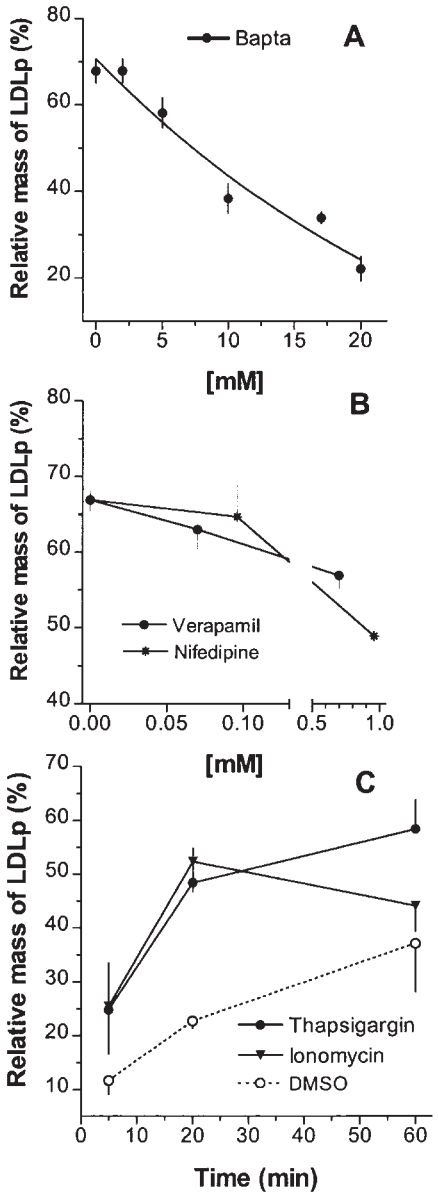

Fig. 3. A) Effect of Bapta on AKH action. Insects were injected with varying concentrations of Bapta. After 20 min, insects were treated with 100 pmol AKH. Thirty min after the AKH injection, hemolymph was collected, pooled, and analyzed for LDLp content. B) Effect of calcium channel blocker nifedipine and verapamil on AKH action. Insects were injected with varying concentrations of nifedipine and verapamil, respectively. After 20 min, insects were treated with 100 pmol AKH. Thirty min later, hemolymph was collected, pooled, and analyzed for LDLp content. C) Time dependence of effects of ionomycin and thapsigargin on DG production. Five μl of 2.7 mm ionomycin, 0.6 mm thapsigargin, and DMSO were injected, respectively, into different groups of insects. At the indicated times after the injection, the hemolymph was collected, pooled, and analyzed for LDLp content. For each condition, hemolymph from at least three insects was pooled and analyzed for LDLp content. Four pools were analyzed with each of the chemical tested at every concentration. Results are the mean ± SEM (n = 4).

leases calcium from cellular stores bypassing the normal physiological process (24, 25).

The effects of 45 μm ionomycin, 10 μm thapsigargin and 1.7% v/v DMSO (control) on the time-courses of the levels of hemolymph DG are shown in Fig. 3C. These Ca^{2+}-mobilizing agents as well as all the water-insoluble drugs used in this work were dissolved in DMSO, a solvent that is highly membrane permeable and is also known to have diverse effects on cells (26, 27). In our case, long-term exposure (120 min) of the fat body to high concentration of DMSO (6.0% v/v) resulted in significant lipid mobilization (data not shown). However, as it can be seen in Fig. 3C, this nonspecific effect was controlled by diminishing the final concentration of DMSO in the hemolymph to 1.7% v/v (5 μl injection) and using a short-term treatment, which in all cases was 20 min. All the water-insoluble compounds were injected under these conditions and controls were always carried out. Consequently, the change between a certain concentration of compound and control reflects solely the effect of that particular compound because in both conditions the same amount of DMSO was injected.

Ionomycin rapidly stimulated the release of DG and large changes in the LDLp concentration were observed. As Fig. 3C shows, a 115% increase in LDLp was already induced 5 min after the treatment whereas a very significant change in LDLp, 130% ($P = 0.007$), compared to control insects was induced at 20 min. If the stimulation of lipid mobilization by ionomycin occurs via a calcium-mediated pathway, a similar response should be elicit by another calcium-mobilizing agent. As with ionomycin, the secretion of DG induced by thapsigargin reached a peak within 5 min (110% activation) and turned very significant, 113% ($P = 0.018$), after 20 min as well. Moreover, pretreatment of the insects with 20 mm Bapta, which chelates hemolymph calcium, proved to inhibit almost completely the lipid mobilization induced by 10 μm thapsigargin (data not shown). This confirmed the dependence of the effect of thapsigargin upon calcium.

Effect of AKH on calcium influx

The results from the preceding experiments suggest that a rise in intracellular calcium is associated with the effect of AKH on lipolysis activation. In order to confirm this possibility, unidirectional calcium influx was measured (17) using in vitro fat body incubations. The uptake of calcium by the fat body was measured after the addition of trace amounts of ^{45}Ca into the medium containing 1 mm of calcium. Measurement of ^{45}Ca uptake was done within 135 sec after the addition of the tracer. This period is short enough to prevent significant labeling of intracellular Ca^{2+}, therefore the efflux of radioactive calcium can be ignored (17). **Figure 4** shows the effect of AKH on the time courses of calcium uptake by the fat body. The hormone caused stimulation in calcium influx that was evident as early as 45 sec after the addition. An increase of calcium influx, which nearly doubled the basal rate, was observed at 135 sec ($P = 0.011$).

Effect of agents that activate lipolysis on A-kinase activity ratio

The effect of some of the compounds that elicited an increase in lipid mobilization on A-kinase activity was investigated. As shown in **Table 1**, treatment with cholera

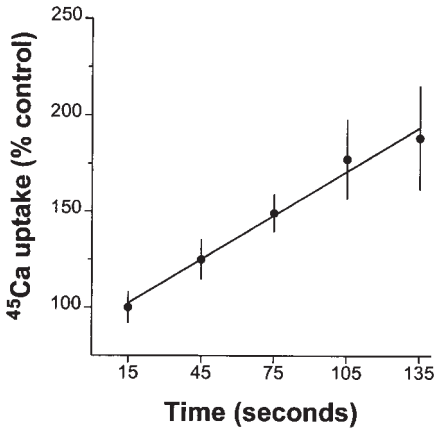

Fig. 4. Effect of AKH on uptake of $^{45}Ca^{2+}$ into fat body as a function of time. Half-fat bodies were incubated in Grace's medium containing 1 mm Ca^{2+}, as indicated described under Materials and Methods. Trace amounts of $^{45}Ca^{2+}$ were added into one piece of tissue, whereas $^{45}Ca^{2+}$ plus 0.4 μm AKH were added into the second half of fat body at zero time. At the indicated time, incubations were terminated and radioactivity associated to the tissue was counted. Results are presented as percent of basal $^{45}Ca^{2+}$ uptake. Each point is the mean ± SEM. (n = 6–8). The line drawn was determined by linear-regression analysis. The correlation coefficient was 0.986.

toxin led to the expected increase in A-kinase activity ratio. A 140% ($P = 0.019$) increase was detected 20 min after cholera toxin treatment. Likewise, both calcium-mobilizing agents induced A-kinase activation. A 72% ($P = 0.048$) and 97% ($P = 0.009$) increase in the A-kinase activation were obtained 20 min after treatment with 10 μm thapsigargin or 45 μm ionomycin, respectively (Table 1).

In order to examine the relationship between the A-kinase activity ratio and the stimulation of lipid mobilization, the data on hemolymph LDLp content and fat body A-kinase activity ratio that were compiled from the different experiments shown in Table 1 were plotted in **Fig. 5**. The line in the figure shows a computer fit of the data to a general sigmoidal equation in which the correlation coefficient, r^2 was 0.8020. As shown in Fig. 5, a sharp transition between the DG production and the activation of A-kinase was obtained. The LDLp level varied from 20% to 50% as the A-kinase activity ratio changed from a value of 0.03 to 0.12, representing about a 300% increase in A-kinase activity.

DISCUSSION

The present results confirm and extend the evidence in support of a dual second messenger system in AKH activation of lipid mobilization in the insect fat body. The results shown in Fig. 1 demonstrate for the first time that AKH induces a rapid and sustained increase in the A-kinase activity of fat body cells, strongly suggesting that AKH produces an increase in the intracellular concentration of cAMP. Further support for a second messenger role of cAMP in lipolysis activation is provided by the data shown in Fig. 2 and Table 1. Forskolin, 8Br-cAMP, and cholera toxin, agents which raise intracellular cAMP, stimulated lipid mobilization, whereas the non-active form of forskolin, 1,9-dideoxyforskolin, did not induce any change in the lipolytic response.

However, a comparison between the lipid mobilization effect produced by agents that raise the intracellular cAMP with the lipolytic effect of AKH showed differences in the magnitude of the responses. After 20 min, lower values of LDLp (32% to 44%) were achieved after treatment with these agents than produced by a maximal dose of AKH (56%). At least two different reasons could explain this result. First, the intracellular concentration of the agent was not sufficient to induce a maximal response. Agents were injected into the hemolymph but the intracellular concentrations are not known. Because the hemolymph is a complex medium with a very high protein concentration, a much lower intracellular concentration than expected could be attained if the agent binds to any of the hemolymph com-

TABLE 1. Effect of agents that stimulate lipid mobilization on A-kinase activity ratio

	PKA Activity Ratio × 10^2		LDLp (%)	
Treatment[a]	0 min	20 min	0 min	20 min
AKH (0.3 μm)	4.8 ± 0.2[b]	17.1 ± 0.6[c]	16.7 ± 2.1	56.2 ± 13.2
Cholera toxin (0.25 μg/μl)	5.1 ± 1.5[d]	12.4 ± 1.2[e]	19.0 ± 2.6	40.8 ± 6.5
	5 min	20 min	5 min	20 min
DMSO (1.7% w/v)	1.0 ± 0.5	3.6 ± 0.7[f]	11.7 ± 2.7	22.7 ± 1.4
Thapsigargin (10 μm)	2.1 ± 0.5	6.2 ± 0.6[g]	24.8 ± 8.7	48.4 ± 6.5
Ionomycin (45 μm)	10.9 ± 0.4	7.1 ± 0.2[h]	38.0 ± 1.4	52.3 ± 5.7

Fat bodies and hemolymph were taken from each insect at the indicated period of time after the treatment. A-kinase activity and LDLp content were determined in fat body homogenates and hemolymph, respectively. Material from three insects was pooled for each measurement. Results represent mean ± SEM (n = 3).

[a] Concentration values in parentheses represent the final concentrations reached in the insect hemolymph that was calculated as indicated in Materials and Methods.

[bc] $P < 0.025$.

[de] $P < 0.025$.

[fh] $P < 0.025$.

[fg] $P < 0.05$.

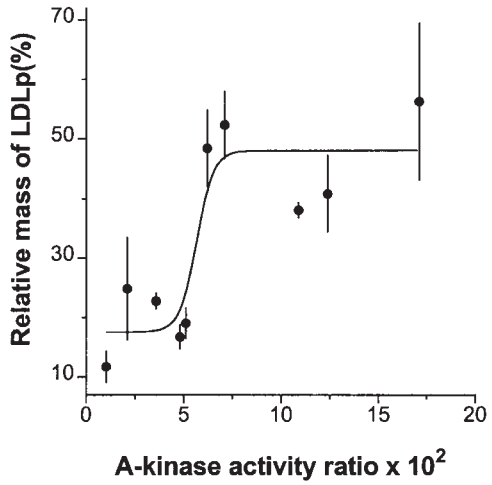

Fig. 5. Relationship between A-kinase activity ratios and production of DG. The data were compiled from the different experiments shown in Table 1. The line in the figure shows a computer fit of the data to a general sigmoidal equation in which the correlation coefficient, r^2 was 0.8020.

ponents. This possibility could account for the effect of 8Br-cAMP in which the lipid mobilization increased in a concentration–response manner. Perhaps, injecting even higher concentrations of this agent, a maximal effect could have been reached. Alternatively, with the use of in vitro fat body culture, we might overcome this potential problem. However, for reasons that remain unclear, the mobilization of DG in vitro occurs with a very low efficiency compared to that occurring in vivo.

The second reason why agents that raise intracellular cAMP did not induce a maximal lipid mobilization response could be related to the existence of an additional second messenger in the action of AKH. In the case of forskolin, the highest response was achieved at the lowest concentration injected. Therefore, at least in this case, it seems more likely that the maximal response was not attained because this agent by itself is not a sufficient stimulus, rather than because the intracellular concentration was too low. A similar result was observed in locust fat body in which in vitro incubation with forskolin induced release of DG but did not achieve the maximal response. Two-fold increase in the synthesis of DG was induced by 10 μm forskolin whereas the maximal response attained by 0.1 μm AKH implicated a 8-fold increase in DG (14).

The effect of AKH in mobilizing lipids was markedly diminished in the presence of a calcium chelator that shows calcium is essential for the lipid mobilization induced by AKH. Further evidence for a second messenger role of Ca^{2+} in AKH action was provided by the experiments with ionomycin and thapsigargin. The effects of these two separate calcium-mobilizing agents proved that an increase in intracellular calcium in the insect fat body leads the stimulation of lipid mobilization. Unlike the response to agents that raise intracellular cAMP, the magnitude of the lipolytic response induced by these agents was similar to the AKH response. These results showed a direct causative effect of Ca^{2+} on stimulation of the lipid mobilization. In keeping with this conclusion is the demonstration that AKH causes a rapid increase in the calcium influx rate into the fat body (Fig. 4). A similar activation of Ca^{2+} influx was reported in isolated rat liver cells by the action of the calcium-mobilizing hormones noradrenaline, vasopressin, and angiotensin and in glomerulosa cells induced by adrenocorticotropin (ACTH) (18, 28). In both reports, the increase of calcium uptake was observed in the same time frame as reported here (18, 28). At this time, it is not possible to distinguish whether the increase in Ca^{2+} influx was due to a direct stimulation of AKH on calcium entry or a secondary consequence resulting from the depletion of the internal stores initiated by AKH. In any event, this experiment confirmed the dependence of AKH action on external calcium, which strongly indicates the involvement of Ca^{2+} as a second messenger in the activation of the lipolytic pathway in the insect fat body. The fact that ionomycin mobilized lipids into the hemolymph is in agreement with previous results in which the calcium ionophore A23187 exhibited a similar effect in the locust fat body. Also, no lipid mobilization was obtained by a massive dose of AKH after the removal of calcium from the incubation medium (13–15).

In common with AKH, the secretion of DG into the hemolymph stimulated by cholera toxin, DMSO, thapsigargin and ionomycin was also accompanied by an increase in the A-kinase activity (Table 1). In addition, a relationship between the production of DG and the rate of A-kinase activity was observed (Fig. 5). These facts strongly indicate that the regulation of the production of DG is through reversible phosphorylation / dephosphorylation, and that activation of the mechanism is associated with protein phosphorylation, as has been shown to occur in the hormonal regulation of HSL (29). Interestingly, a very similar relationship between the A-kinase activity ratio and lipolysis was observed in adipocytes, where glycerol production varies from nil to maximal values as the A-kinase activity ratios varied from 0.05 to 0.35 (17). This type of dependency was interpreted as the amplification of the response, which means that one molecule of A-kinase will phosphorylate more than one molecule of the substrate (17).

Adipokinetic hormones together with the red pigment-concentrating hormone from crustacea and the hypertrehalosemic hormones of insects are a very large family of 8–10 amino acid peptides. In general, the most widely recognized action by these peptides is their stimulation of the fat body to convert stored TG or glycogen into hemolymph DG or trehalose, respectively, which serve for use as sources of energy. In the case of *M. sexta*, AKH stimulates mostly glycogenolysis in the feeding larval stage and lipid mobilization in the non-feeding adult stage (30, 31).

In vertebrates, the cAMP system is of major importance for the regulation of lipolysis in fat cells where catecholamines play a central role in promoting lipolysis. This effect is mediated by β-adrenergic receptor–adenylate cyclase complex without affecting cytosolic calcium concentration. The receptor-controlled increase in intracellular cAMP concentrations promotes activation of A-kinase,

which phosphorylates a serine residue (Ser-563 for the rat and Ser-551 for the human) in HSL and promotes its activation and its translocation towards the lipid droplet (32). On the other hand, catecholamines, via an α_1-adrenoreceptor, initiate phosphoinositide hydrolysis in white fat cells and elevate cytosolic calcium concentration without raising cAMP. This mechanism results in the stimulation of glycogenolysis (32).

In addition to the evidence in favor of cAMP and Ca^{2+} as second messengers in the action of AKH in mobilizing lipids that are presented in this study and in previous investigations, recent reports showed that AKH increases levels of inositol (1,4,5)-triphosphate (InP_3) in the fat body of two locusts, *Schistocerca gregaria* (33) and *Locusta migratoria* (34). Interestingly, the report on *Schistocerca gregaria* showed a difference in sensitivity between the cAMP and the $InsP_3$ response. The latter exhibited EC_{50} values for AKH that are 100-fold higher than the respective values for the cAMP response.

The sequence of events leading to the stimulation of lipolysis induced by AKH is unknown. The present data support a model in which the binding of AKH to its receptor initiates two events, a rapid and sustained increase in Ca^{2+} influx and an activation of adenylate cyclase, giving rise to two intracellular messenger, Ca^{2+} and cAMP. Given the fact that calcium-mobilizing agents fully mimic the action of AKH in regard to the magnitude of the DG production and the activation of A-kinase, it is reasonable to propose that calcium potentiates the action of cAMP. At present, the substrates for the fat body A-kinase are under investigation. A-kinase purified from the insect fat body phosphorylates the fat body TG-lipase as well as other proteins associated with the fat droplets (E. L. Arrese and M. A. Wells, unpublished results). Likely, the activation of A-kinase induced by AKH results in the phosphorylation of TG-lipase, which might lead to its activation and/or translocation to the substrate stores in the fat droplets.

In vertebrate adipose tissue, the intracellular concentrations of calcium and cAMP are controlled by separate receptors. Little is known about the AKH receptor. In *Manduca sexta*, the main target tissue for AKH is the fat body and Scatchard analysis of specific binding suggested only one type of receptor (35). If this is the case, then the appearance of one of these two messengers should precede the other. In mammalian tissues, different patterns of cross-talk between the Ca^{2+} and cAMP signal transduction systems have been established. For example, intracellular free calcium can affect cAMP level by modulation of adenylate cyclase activity or phosphodiesterase activities (36, 37). On the other hand, A-kinase or cAMP can affect Ca^{2+} by regulating Ca^{2+} ion channel activity (38). Given the fact that the magnitude of the lipolytic response induced by Ca^{2+}-mobilizing agents was similar to the AKH response, we suggest a model in which AKH first raises the intracellular calcium concentration, which in turn activates adenylate cyclase. However, a new form of cross-talk between these two regulatory systems could also occur in the insect fat body.

In conclusion, unlike the vertebrate system in which lipolysis is regulated solely by the cAMP system, in the insect fat body, both cAMP and calcium are relevant messengers in the stimulation of *sn*-1,2-DG production. Although the results seem to indicate that calcium plays a primary role in the signal pathway of AKH, further experiments are required in order to dissect the cross-talk between these two second messengers. Finally, the activation of A-kinase could be involved in the phosphorylation of the TG-lipase, the enzyme that catalyzes the conversion of TG into *sn*-1, 2-DG.

We thank Dr. Jose L. Soulages for critical review of this manuscript and Mary Hernandez for animal care. This work was supported by NIH grant GM 51296. The Undergraduate Biology Research Program of the University of Arizona supported M. F. and J. G.

Manuscript received 4 May 1998, in revised form 22 September 1998, and in re-revised form 11 November 1998.

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