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Developmental changes in the response of larval *Manduca sexta* fat body glycogen phosphorylase to starvation, stress and octopamine

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Abstract

Fasting or starvation of 1st- and 2nd-day fifth instar *Manduca sexta* larvae leads to rapid activation of fat body glycogen phosphorylase. Under feeding conditions, 21–29% of the phosphorylase was found in the active form. However, after only one hour of starvation, the active form increased to 55–65%. In larvae on the 3rd-day there was a slower increase in the activation, requiring three hours of starvation to reach a maximum of 60–65%. No activation was observed in 4th-day larvae after three hours of starvation. When 1st- or 2nd-day larvae were decapitated, the time-course of activation of glycogen phosphorylase was very similar to that observed in intact insects. However, activation of glycogen phosphorylase following decapitation was only observed in 1st- and 2nd-day larvae. In 2nd-day larvae, octopamine promoted activation of glycogen phosphorylase and 100-pmol of octopamine promoted maximum activation. Higher amounts of injected octopamine caused a decrease in activation. The injection of 100 pmol of octopamine caused a 50–55% activation of phosphorylase within 30 minutes. The simultaneous injection of the α -adrenergic receptor antagonist phentolamine with octopamine blocked the octopamine effect in 1st- and 2nd-day feeding larvae. However, the activation of glycogen phosphorylase observed in ligated/decapitated larvae on the 1st- and 2nd-day was not abolished by injection of phentolamine. All of these data suggest that factors other than adipokinetic hormone and octopamine may be involved in the activation of glycogen phosphorylase during fasting or starvation in the early part of the fifth larval stage of *M. sexta*. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Fat body; *Manduca sexta*; Glycogen phosphorylase; Octopamine; Stress; Adipokinetic hormone

1. Introduction

In insects two different types of hormones have been implicated in the mobilization of fat body energy stores: the adipokinetic hormones (AKHs), which are a family of structurally related peptides containing 8–10 amino acids that occur widely in insects (Orchard, 1987, Gäde, 1990), and octopamine, the major biogenic amine found

in invertebrates (Axelrod and Saavedra, 1977), which acts both as a neurotransmitter, modulating the release of AKH from the corpus cardiacum (Passier et al., 1995), and as a hormone with direct energy store mobilizing activity on the fat body (Orchard and Loughton, 1985). Evidence that glycogenolysis in the insect fat body is under hormonal regulation has been reported in many insect species (for a review see Gäde, 1990). Injections of Manduca-AKH (M-AKH) into larvae of *Manduca sexta* activates fat body glycogen phosphorylase (Ziegler, 1990; Ziegler et al., 1991; Siebert 1992, 1995; Siebert and Mordue, 1994), suggesting, but not proving, that M-AKH might play an important role in survival during larval fasting or starvation (Ziegler, 1995). In *M. sexta* the activation of fat body glycogen phosphorylase undergoes characteristic changes during the late stages of larval development (Siebert, 1987a; Siebert et al., 1993) and starvation (Siebert and Ziegler, 1983; Siebert,

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1987b). Thus, Ziegler (1984) and Siegert and Mordue (1994) showed that the response to injections of extracts from corpora cardiaca or AKH depends on the age of the animal during late larval development. Here we show that activation of glycogen phosphorylase during starvation in the first 48 h of the last larval stage of *M. sexta* does not depend on AKH or octopamine.

2. Material and methods

2.1. Chemicals

D,L-octopamine, AMP, glycogen, phosphoglucomutase, glucose-6-phosphoglucomutase, glucose-6-phosphate dehydrogenase and coenzymes were obtained from Sigma Chemical Co. (St Louis, MO). [14]-C-inulin was obtained from Amersham Life Science Inc. (Arlington Heights, IL).

2.2. Insects

M. sexta were raised as previously described (Prasad et al., 1986). Animals were synchronized at the end of the fourth larval instar by the appearance of head capsule apolysis (Truman et al., 1973). For most of the experiments, intact and fed animals were used, but in some experiments larvae were ligated with dental floss between the second and third pair of prolegs. Ligations were done at this position so that the anterior and posterior sections would have approximately equal amounts of fat body. Ligated larval abdomens were used to remove the influence of the brain, corpora cardiaca, corpora allata and prothoracic glands (Siegert, 1992). During starvation studies fecal pellets were removed to prevent coprophagy.

2.3. Determination of the fat body glycogen phosphorylase activity

Larvae were pinned in a dish and cut open along the dorsal midline. Fat body was washed with 0.15 M NaCl

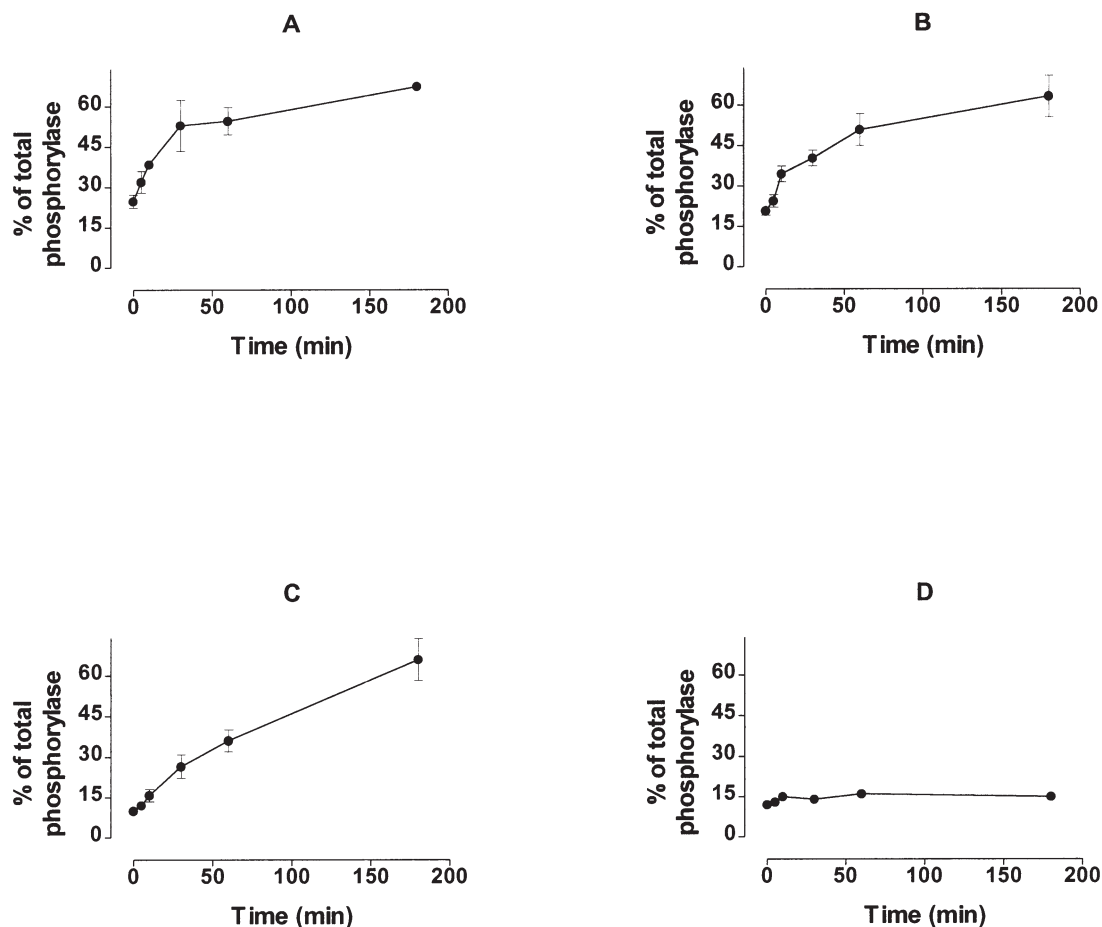


Fig. 1. Time-course of activation of the glycogen phosphorylase in starved fifth instar larvae: (A) 1st day, (B) 2nd day, (C) 3rd day and (D) 4th day. The response to starvation was measured as the percentage of the total phosphorylase activity present as phosphorylase a. Values given are means \pm S.E.M. of three independent experiments using three different insects each.

and removed. The fat body cells were disrupted by homogenization in a buffer containing 50 mM MOPS–TRIS, pH 7.2, 5 mM NaF, 5 mM EDTA, 1 mM EGTA, and 1 mM dithiothreitol (DTT). The homogenate was centrifuged (20 min, 12,000 *g*) and the supernatant used for determination of glycogen phosphorylase activity in the direction of glycogen breakdown by coupling the production of glucose-1-phosphate to the reduction of NADP using phosphoglucomutase and glucose-6-phosphate dehydrogenase. Standard conditions (Arrese et al., 1995) for measurement of glycogen phosphorylase activity were: 40 mM phosphate buffer (pH 7.0), 5 mM imidazole, 2 mM NaEDTA, 1.4 mM dithiothreitol (DTT), 5 mM magnesium acetate, 4 μ M glucose-1, 6-bisphosphate, 0.6 mM NADP, 2 mg/ml glycogen (free from AMP), 4 U of phosphoglucomutase, and 0.8 U of glucose-6-phosphate dehydrogenase. Thirty microliter aliquots of the supernatant fraction obtained from the fat body homogenate were assayed for active phosphorylase in the absence of 5'-AMP (phosphorylase a activity), and for total phosphorylase activity in the presence of 2 mM 5'-AMP (Arrese et al., 1995). Values for the active phosphorylase a activity are expressed as the percentage of total phosphorylase activity. Endogenous NADP-dehydrogenase activity (e.g., glucose-6-phosphate dehydrogenase) that is present in the homogenate supernatant resulted in NADP reduction that must be corrected for by a blank made without adding enzymes. If this correction is not made, the activation of phosphorylase in the supernatant is overestimated (Arrese et al., 1995). All experiments were done three times using fat body from three different insects in each experiment.

2.4. Hemolymph volume determination

[¹⁴C]-inulin (Amersham Life Science Inc., Arlington Heights, IL, 1.74 μ Ci/mg) dissolved in PBS (10 mM potassium phosphate, 0.15 M NaCl, pH 6.5) was used to determine the volume of the hemolymph. The insects were weighed and 5–7 μ l of a freshly prepared [¹⁴C]-inulin solution (90,000–300,000 dpm) was injected into the fourth pair of prolegs of each animal using a 10 μ l Hamilton syringe. Ninety minutes later, hemolymph was collected into a tube containing a few crystals of phenylthiourea from a puncture in the first pair of prolegs. An aliquot (50 μ l) was taken, and added to 10 ml of liquid scintillation cocktail (Budget-Solve, RPI, Mount Prospect, IL) to determine the radioactivity. The volume of hemolymph was determined according to the dilution of the injected ¹⁴C-inulin. For insects starved for 3 h, ¹⁴C-inulin was injected 90 min after the onset of starvation, and 90 min later hemolymph was collected. The insects were weighed before and at the end of the 3 h-starvation treatment.

2.5. Statistics

Differences were evaluated for statistical significance using Student's *t*-test.

3. Results and discussion

3.1. Effects of starvation in *Manduca sexta* larvae

It has been suggested that the activation of fat body glycogen phosphorylase may undergo developmental changes in *M. sexta* (Ziegler, 1984; Siegert and Mordue, 1994). However, the activation of glycogen phosphorylase during starvation had only been studied in 3rd-day fifth instar larvae (Siegert, 1987b; Gies et al., 1988). In 1st- and 2nd-day fifth instar larvae the activation of glycogen phosphorylase during starvation is very rapid (Fig. 1, panels A and B), increasing from 21–29% active phosphorylase in the feeding stage to 55–65% active phosphorylase after 1 h of starvation. In larvae on the 3rd-day, there was a slower increase in activation of phosphorylase, requiring 3 h of starvation to reach a maximum of 60–65% (Fig. 1, panel C)—this value was maintained until 6 h. After this period a decrease in activation of phosphorylase was observed (data not shown). No activation of phosphorylase was observed after 3 h of starvation in larvae of the 4th-day (Fig. 1, panel D).

M. sexta larvae feed nearly constantly day and night, pausing only to molt (Reinecke et al., 1980). However, larvae from different days do stop eating for short periods of time. First and second-day larvae can be observed to stop feeding for 15–45 min, which is enough time to cause activation of glycogen phosphorylase (Fig. 1, panels A and B). Therefore, in these experiments, only larvae that had been actively feeding for at least 5 min

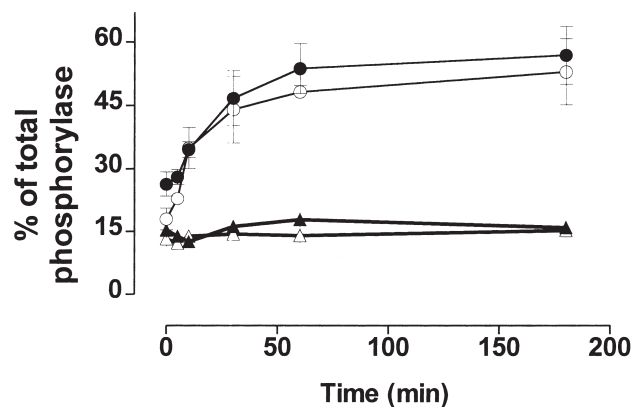


Fig. 2. Time-course of activation of the glycogen phosphorylase in decapitated fifth instar larvae: 1st day, (●) 2nd day, (○) 3rd day and (▲) 4th day (△). The response to decapitation was measured as the percentage of the total phosphorylase activity present as phosphorylase a. Values given are means \pm S.E.M. of three independent experiments using three different insects each.

were selected for starvation studies, because it has been shown that after starvation, refeeding for 2–5 min is long enough to inactivate fat body glycogen phosphorylase (Siegert and Mordue, 1992).

3.2. Activation of glycogen phosphorylase in ligated/decapitated larvae

It has been proposed that AKH, at least in part, controls carbohydrate metabolism during starvation/fasting in larval *M. sexta* (Siegert and Ziegler, 1983; Ziegler et al., 1990; Siegert and Mordue, 1992; Ziegler, 1995). Thus, when 4th-day fifth instar larvae (9–10 g body wt) were injected with 20 pmol M-AKH they showed activation of glycogen phosphorylase (Siegert and Mordue, 1994), even though larvae of this age showed no activation of glycogen phosphorylase during a short time of starvation (Fig. 1, panel D). In the experiments shown in Fig. 2, feeding larvae from different days were ligated and decapitated, and the activation of the glycogen phosphorylase was studied. Activation of glycogen phosphorylase was only observed in decapitated 1st- and 2nd-day larvae, with a time-course of activation very similar to that observed during starvation of whole insects (Fig. 1, panels A and B). Thus, 1 h after ligation/decapitation, glycogen phosphorylase activation increased to a maximum. Interestingly, decapitation of larvae on the 3rd- and 4th-days did not cause activation of glycogen phosphorylase after 3 h (Fig. 2). These data suggest that in larvae on the 1st- and 2nd-days, factors other than AKH were responsible for the activation of glycogen phosphorylase.

3.3. Effect of stress and octopamine on glycogen phosphorylase activation

The activation of glycogen phosphorylase observed in decapitated 1st- and 2nd-day larvae could result from fac-

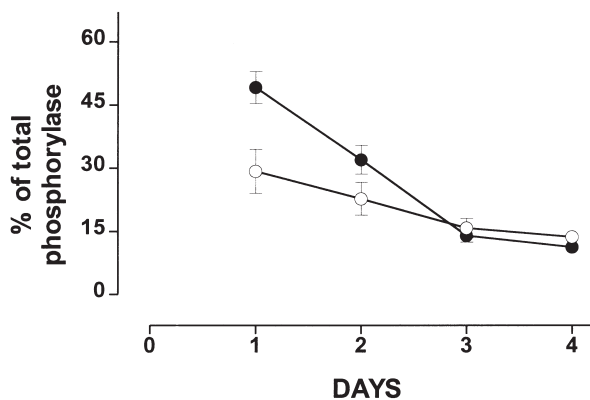


Fig. 3. Effect of injection of water on activation of the glycogen phosphorylase in fed fifth instar larvae. Injection of 10 μ l of water (●), without injection (○). The response to injection of water was measured as the percentage of the total phosphorylase activity present as phosphorylase a. Values given are means \pm S.E.M. of three independent experiments using three different insects each.

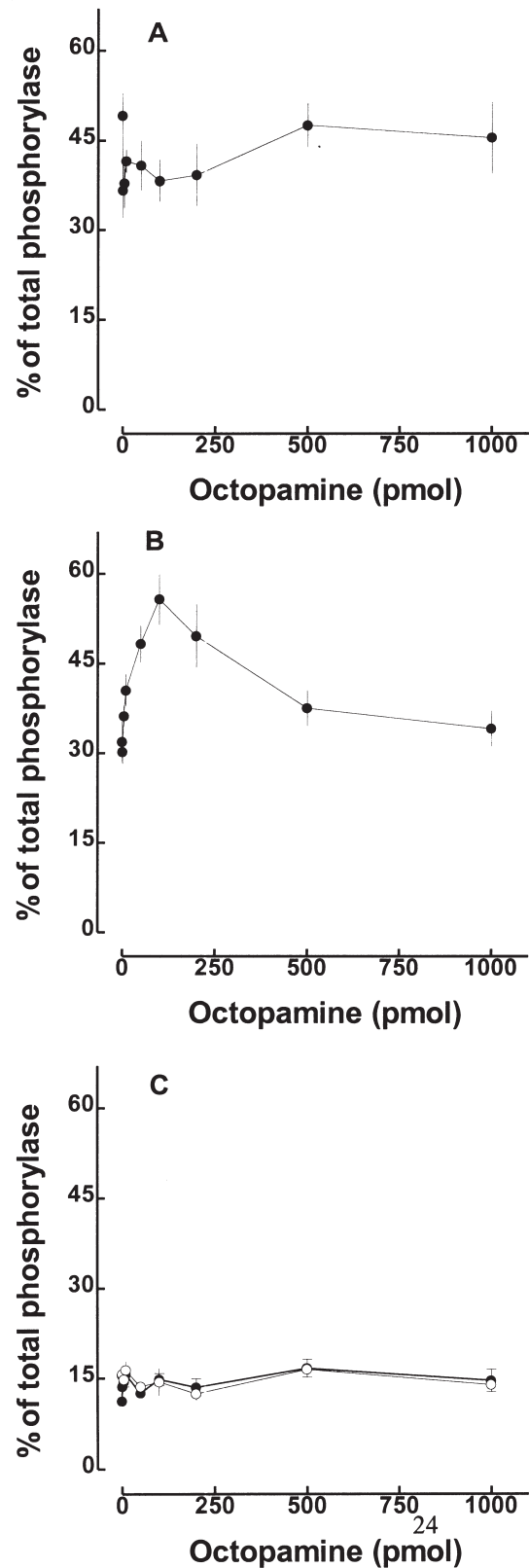


Fig. 4. Effect of increasing amounts of octopamine injected on activation of the glycogen phosphorylase in fed fifth instar larvae: (A) 1st day, (B) 2nd day, (C) 3rd (●) and 4th (○) days. The response to injection of octopamine was measured as the percentage of the total phosphorylase activity present as phosphorylase a. Values given are means \pm S.E.M. of three independent experiments using three different insects each.

tors related to stress (starvation) that are released from sources other than the head. In the cockroach (Matthews and Downer, 1974) and locust (Orchard et al., 1981) it was shown that the hyperglycemic and hyperlipemic responses are increased during stress conditions, and that octopamine is involved in both processes. In both insects (Davenport and Evans, 1984a) an increase in hemolymph octopamine levels is a general response to different forms of stress. Food deprivation also induces an increase in octopamine levels in the hemolymph of locusts (Davenport and Evans, 1984b) as well as in *M. sexta* (Adamo et al., 1997). Ismail and Matsumura (1992) showed that injections of octopamine elevated glucose and trehalose levels in the hemolymph of *M. sexta*. It has been observed that hemolymph trehalose is homeostatically regulated at the expense of tissue glycogen during starvation (Murphy and Wyatt, 1965; Steele and Hall, 1985). To test the possible effects of octopamine in the activation of glycogen phosphorylase in *M. sexta*, it was necessary to inject the animals, but as shown in Fig. 3, the injection of water into 1st- and 2nd-day larvae activated glycogen phosphorylase, whereas in 3rd- and 4th-day larvae no activation was observed.

Because the hemolymph volume of a 4th-day larva is four to five times higher than a 1st day larva, we injected 50 μ l of water into 3rd- or 4th-day larvae, but found no effect (data not shown). These results again point out the differences in response between early and late fifth instar larvae. In addition, no changes in the volume of hemolymph were observed during starvation, even after 24 h (data not shown) and the volumes determined were similar to those previously reported (Beckage and Riddiford, 1982).

It was necessary to distinguish the response to injections of water (Fig. 3) from that due to the injected octopamine. In this case, it was not possible to use ligated larval abdomens because, as observed in Fig. 2, in 1st- and 2nd-day larvae the removal of the head did not eliminate the rapid activation of glycogen phosphorylase, therefore we used feeding animals for these experiments. The dose response to octopamine is shown in Fig. 4. In larvae on the 1st-day (Fig. 4, panel A), it was not possible to distinguish between the responses to injections of water (close circles) and octopamine (open circles). It seems that on this day the response to stress (Fig. 3) is higher than on the other days, and it is possible that

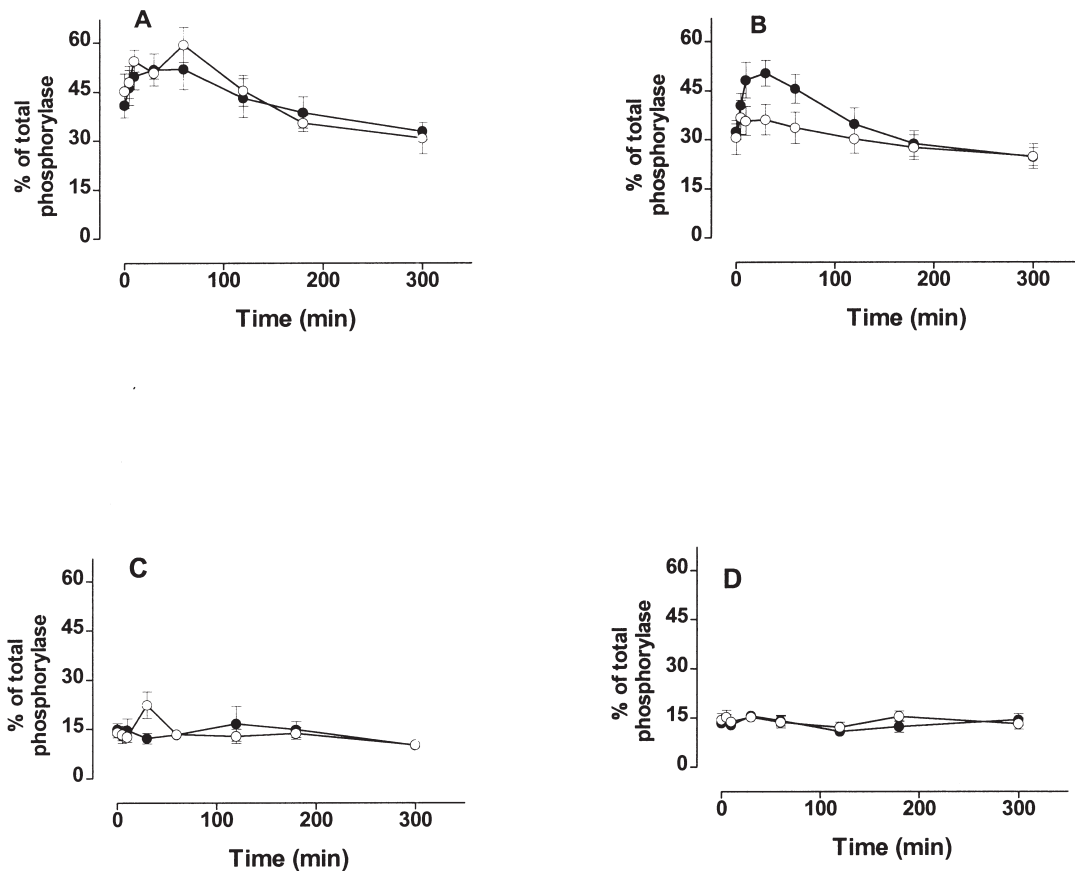


Fig. 5. Time-course of activation of the glycogen phosphorylase in fed fifth-instar larvae to injections of octopamine: 1st day, (A) 2nd day, (B) 3rd day and (C) 4th day larva (D). (●) 100 pmol octopamine and (○) water. The response to injection of octopamine was measured as the percentage of the total phosphorylase activity present as phosphorylase a. Values given are means \pm S.E.M. of three independent experiments using three different insects each.

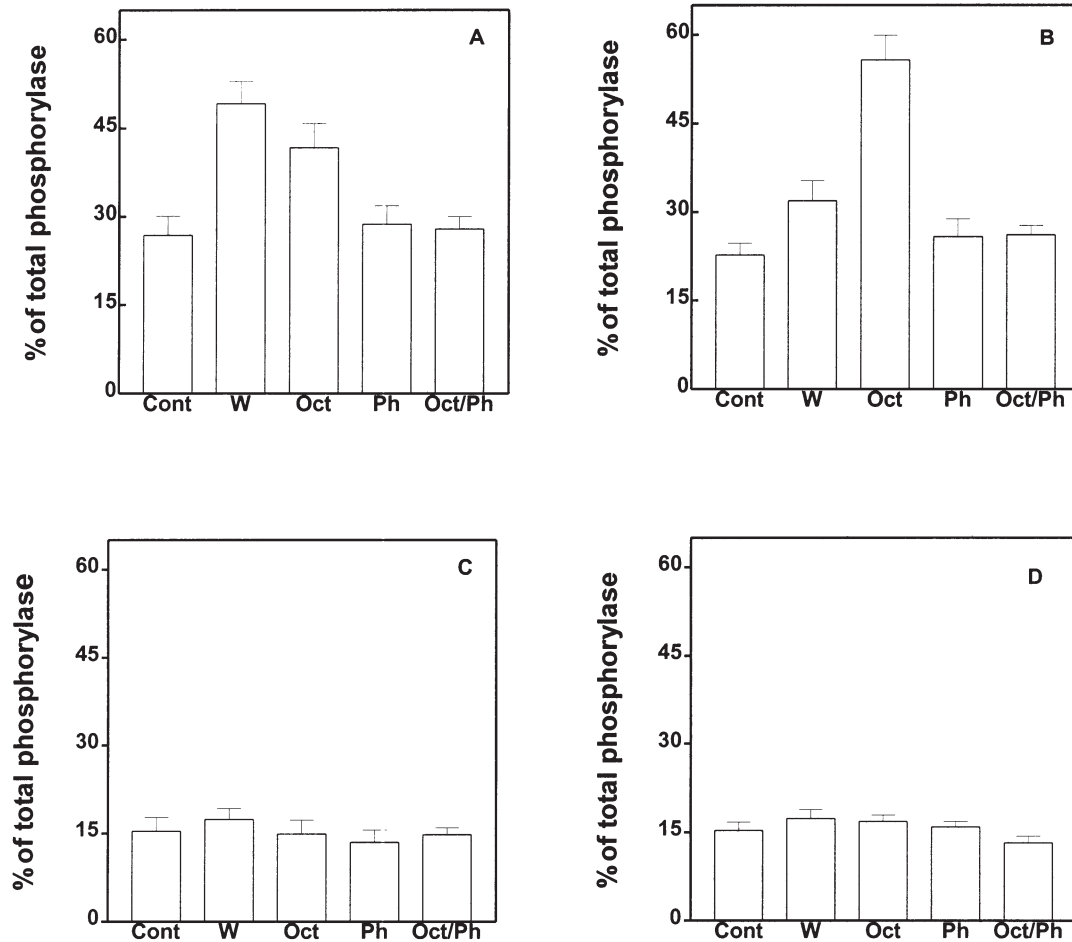


Fig. 6. Effects of water, octopamine and phentolamine on the activation of glycogen phosphorylase in fed fifth instar larvae. 1st day, (A) 2nd day, (B) 3rd day and (C) 4th day (D) larva were injected with either water (W); 100 pmol of octopamine (Oct); 100 pmol of phentolamine (Ph); or 100 pmol each of octopamine and phentolamine. The response was measured as the percentage of the total phosphorylase activity present as phosphorylase a. Control (Cont) insects were not injected. Values given are means \pm S.E.M. of three independent experiments using three different insects each.

endogenous octopamine is released by the injection of water and it may be enough to promote the maximal activation of glycogen phosphorylase.

Second day larvae are less sensitive to injection of water (Fig. 3), and it was possible to observe the activation of glycogen phosphorylase promoted by octopamine (Fig. 4, panel B, open circles). Interestingly, the dose response for octopamine showed two components (stimulatory and inhibitory). The injection of 100-pmol octopamine promoted maximal activation. In the second day larvae (1.24 ± 0.37 ml of hemolymph) this amount of octopamine corresponded to 80.6 ± 24.0 nM in the hemolymph, which is in the same range of concentration of circulating octopamine (around 130 nM) reported for *M. sexta* under starvation conditions (Adamo et al., 1997), and for *Schistocerca gregaria* and *Periplaneta americana* (around 80–100 nM) under thermal stress conditions (Davenport and Evans, 1984a). Above this concentration a decrease in the activation of glycogen phosphorylase was observed (Fig. 4, panel B). This result might suggest that in *M. sexta* the octopamine

effect could be under feedback regulatory control. Fields and Woodring (1991) have suggested that high sugar levels might inhibit octopamine-dependent carbohydrate mobilization from the fat body in *Acheta domesticus*. Octopamine did not activate glycogen phosphorylase in 3rd- and 4th-day larvae (Fig. 4, panel C). These larvae also did not respond to starvation, injection of water, or decapitation.

3.4. The time-course of the effect of octopamine

Fig. 5 confirmed that only in 2nd-day larvae it was possible to observe activation of glycogen phosphorylase after octopamine injections (Fig. 5, panel B), reaching a maximal value in 30 min. The short-lasting effects of octopamine could be due to its rapid removal from the circulation. In locusts it has been shown that injected octopamine is rapidly removed from the hemolymph (Goosey and Candy, 1982). This Figure also shows that the kinetics of enzyme activation by water injection follows the same time course as that caused by injection

of octopamine. Although in 1st-day larvae it was not possible to observe any difference between injections of water and octopamine (Fig. 5, panel A), the time-course for activation and inactivation was very similar to that observed in larvae on the 2nd-day after injections of octopamine (Fig. 5, panel B). In the larvae on the 3rd and 4th days the injection of 100-pmol of octopamine failed to activate glycogen phosphorylase (Fig. 5, panels C and D).

It has been shown in *A. domesticus* and *M. sexta* that the response to adipokinetic hormones in lipid mobilization and glycogen phosphorylase activation depends on the age of insect (Woodring et al., 1990; Siegert and Mordue, 1994). Fields and Woodring (1991) showed that octopamine-induced mobilization of lipids and carbohydrates in adult of *A. domesticus* also depends on the age of the insect. Our results show that any effect by octopamine on activation of glycogen phosphorylase during the last larval stage *M. sexta* also depends on the age of the insect. The level of glucose in the hemolymph of 1st- and 2nd-day larvae (3.56 ± 0.45 mM and 3.39 ± 0.29 mM, respectively) under feeding conditions is higher than in 3rd- and 4th-day larvae (1.46 ± 0.23 mM and 1.24 ± 0.18 mM, respectively). Whether the glucose level in the hemolymph is an important factor related to the activation of glycogen phosphorylase remains to be elucidated.

3.5. Effect of phentolamine on the activation of glycogen phosphorylase

The α -adrenergic receptor antagonist phentolamine suppressed the activation of glycogen phosphorylase on the 1st- and 2nd-days caused by water or octopamine injection (Fig. 6, panels A and B). The suppression by phentolamine of the activation of the phosphorylase caused by injection of water (Fig. 6, panel A), suggests that a possible release of octopamine after injection of water could be involved on the activation of the phosphorylase. The injection of phentolamine into 3rd- or 4th-days larvae had no effect (Fig. 6, panels C and D). Larval *M. sexta* octopamine receptors therefore show characteristics similar to octopamine receptors in other insects (Fields and Woodring, 1991). The role of octopamine in energy mobilization has been related to adult stage flight activity (Orchard, 1987). Here we showed that octopamine was able to increase the active form of glycogen phosphorylase in the fat body of *M. sexta* larvae.

The activation of glycogen phosphorylase observed in ligated/decapitated 1st- and 2nd-day larvae (Fig. 2) was not abolished by phentolamine (Fig. 7), suggesting that factors other than AKH and octopamine may be involved in the activation of the glycogen phosphorylase during starvation. Further studies will be required to characterize these factors.

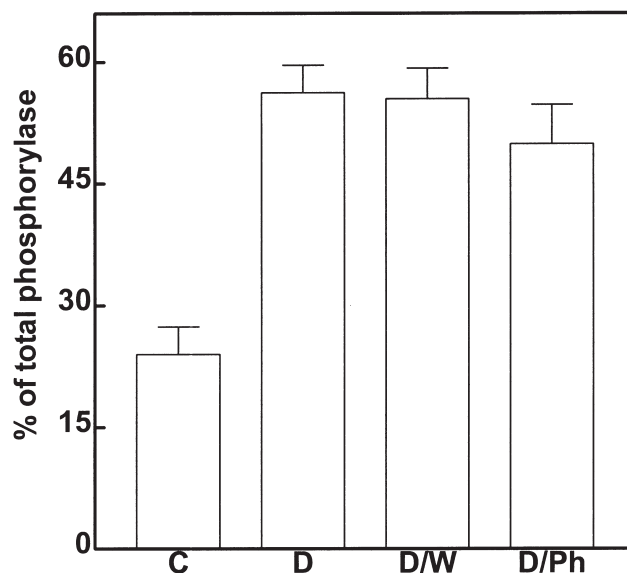


Fig. 7. Effects of water and phentolamine on activation of glycogen phosphorylase in decapitated 2nd day fifth instar larvae. Insects were ligated and decapitated and the activation of glycogen phosphorylase measured 30 min later (D); or insects were ligated and decapitated and injected with either water (D/W) or 100 pmol phentolamine (D/Ph) and the activation of glycogen phosphorylase measured 30 min later. Control insects (C) were not decapitated or injected. The response was measured as the percentage of the total phosphorylase activity present as phosphorylase a. Values given are means \pm S.E.M. of three independent experiments using three different insects each.

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