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INVOLVEMENT OF CYCLIC NUCLEOTIDES IN LOCUST FLIGHT MUSCLE METABOLISM

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Abstract—1. Flight had no significant effect on the levels of c-AMP or c-GMP in the flight muscles of *Locusta migratoria*.

2. Injections of 0.01 or 0.1 corpus cardiacum equivalents into the abdominal cavity did not elicit any effect on cyclic nucleotide levels either.

3. Injection of A23187 resulted in a decrease of the c-AMP level, but not the c-GMP level.

4. Marked increase of the c-AMP level was found to occur in the flight muscles after injection of octopamine, whereas c-GMP level was not influenced.

5. It is concluded that octopamine acts on locust flight muscles through c-AMP mediated activation of glycogen phosphorylase.

INTRODUCTION

Lipid mobilization from the locust fat body is controlled by an adipokinetic hormone (AKH), present in the corpora cardiaca (Beenakkers, 1969; Mayer & Candy, 1969) and released during flight (Rademakers *et al.*, 1976; Robinson & Goldsworthy, 1976). Gäde & Holwerda (1976) have shown that this control is mediated by cyclic AMP: flight performance as well as injection of a corpus cardiacum extract resulted in an increased level of cyclic AMP in the fat body of adult locusts. Injection of corpus cardiacum extract did not elicit a rise in cyclic AMP concentration in the flight muscles.

Robinson & Goldsworthy (1977) demonstrated, however, that corpus cardiacum extract stimulates lipid utilization in locust flight muscles in preference to carbohydrate oxidation. Since in the presence of AKH, pyruvate formation from [^{14}C]glycerol is significantly higher than from [^{14}C]trehalose they suggested that the main point of reduction of the glycolytic flux is situated before the formation of triose phosphates.

Changes in metabolite concentrations in flight muscles of the locust during flight (Worm & Beenakkers, 1979; Worm *et al.*, 1980) indicate positive cross-overs at the hexokinase and probably at the aldolase catalysed reactions and a negative cross-over in the section between glucose-6-phosphate and fructose-1,6-diphosphate. There is, however, no change in this cross-over situation between 5 min of flight, when mainly trehalose is utilized and after 30 min of flight, when mainly diglycerides are utilized.

Recently, Candy (1978) reported that octopamine stimulates glucose and trehalose oxidation in locust flight muscle. This biogenic amine is present in nervous tissue of insects (Robertson & Juorio, 1976). Moreover, David & Lafon-Cazal (1979) reported that besides the central nervous system, also the corpora cardiaca, especially the glandular lobe, contain octopamine.

Because L-glutamate is the main neuromuscular transmitter in locusts and other insects (Lunt, 1975), Candy (1978) supposed that octopamine modulates the response of the muscle to nervous stimulation. However, Evans & O'Shea (1978) found the axons innervating the extensor tibiae muscles of the metathoracic legs of the locust to be octopaminergic and to act directly on the muscle fibers.

Octopamine has been shown to affect cyclic AMP concentrations. Bodnaryk (1979) found an octopamine sensitive adenylate cyclase in the brain of the moth, *Mamestra configurata*. Adenylate cyclases stimulated by low concentrations of octopamine were demonstrated also in nervous tissues of the cockroach, *Periplaneta americana* (Nathanson & Greengard, 1973). These data suggest that the action of octopamine in the insect nervous system may be mediated by cyclic AMP. The intracellular level of cyclic AMP is the resultant of the action of two antagonistic enzymes; the adenylate cyclase and the phosphodiesterase (PDE). Volmer (1977) showed PDE from the flight muscles of *Locusta migratoria* to be specific to cyclic AMP and strongly dependent on the concentration of calcium; these two properties are in marked contrast to vertebrate PDE.

Until now no direct evidence is available that the effects of octopamine on flight muscle metabolism are cyclic nucleotide dependent, while the role of AKH in the regulation of muscle metabolism is inconclusive. Because cyclic GMP was nearly equally potent as cyclic AMP in stimulating fat body protein kinase (Beenakkers *et al.*, 1978) it is conceivable that AKH might exert its effect on flight muscle metabolism by means of cyclic GMP enhancement. Therefore we measured the changes in the concentrations of cyclic AMP and cyclic GMP in the flight muscles of *Locusta migratoria* both during flight and after injections of corpora cardiaca extracts, of octopamine or of the calcium ionophore A23187.

MATERIALS AND METHODS

Insects

Adult males of *Locusta migratoria migratorioides* (12 days after imaginal ecdysis) were used in all experiments. The locusts were reared and flown under conditions described before (Worm & Beenakkers, 1979).

Injections

Extracts of corpora cardiaca (preparation described by Holwerda *et al.*, 1977), solutions of octopamine or of A23187 in insect saline were injected in a 10 μl volume into the abdominal cavity. Octopamine was injected in 10^{-2} or 10^{-5} M solution, giving rise to a final molarity in the haemolymph of approximately 400 or 0.4 μM, as haemolymph volume in *Locusta migratoria* measures about 250 μl.

For the A23187 these figures are 10^{-7} M solution injected and a final concentration of 4 nM.

In the experiments where theophylline was introduced a 0.25 M solution was used, giving a 10 mM concentration in the haemolymph.

Determination of nucleotides

After termination of flight or incubation period the flight muscles were dissected as described before (Worm & Beenakkers, 1979). Dorsolongitudinal muscles of five locusts were pooled for each extract and powdered under liquid nitrogen with pestle and mortar. The powdered tissue was immediately mixed with 4 ml of ice-cold 5% trichloroacetic acid and sonicated for 20 sec.

The homogenates were centrifuged for 15 min at 40,000 ***g***. The pellet was washed with 1 ml ice-cold aqua bidest and centrifuged again for 5 min. To the combined supernatants solid calcium carbonate was added according to the method of Tihon *et al.* (1977) to give a pH of approx 6.0. Complete neutralization was reached after washing the supernatant three times with an equal volume of ether saturated with aqua bidest. After that the samples were buffered with 1 ml Tris–EDTA buffer 50 mM pH 7.5 and used directly for measuring c-AMP and c-GMP. Standards treated in this manner revealed no significant loss of nucleotides.

Haemolymph samples (50 μl) were centrifuged at 2000 ***g*** and the supernatant was mixed with 100 μl 5% TCA, by means of a Vortex mixer and centrifuged again at 2000 ***g***. The samples were neutralized as described for the muscle extracts.

c-AMP and c-GMP were determined according to the method of Gilman (1970) as modified by Brown *et al.* (1971). The assay kits were obtained from Radiochemical Centre, Amersham (United Kingdom). Radioactivities were measured in a Packard liquid scintillation spectrometer (model 2420). Counting efficiency in Emulsifier 299-TM scintillation cocktail (Packard) amounted to about 36%.

Protein determination

Protein was determined according to the method of Schacterle & Pollack (1973) using bovine serum albumine as a standard, after dissolving the pellet obtained by the TCA extraction in 1 M sodium hydroxide and appropriate dilution with aqua bidest.

Statistics

The significance of the results was tested with the Wilcoxon two sample test (de Jonge, 1963).

Chemicals

Cyclic AMP and cyclic GMP were obtained from Boehringer (Mannheim, West Germany). DL-octopamine and theophylline were obtained from the Sigma Chemical Co Ltd (Kingston-upon-Thames, U.K.) and A23187 was a gift from Lilly and Co (Indianapolis, U.S.A.).

Abbreviations

c-AMP Adenosine 3′:5′-cyclic monophosphate; c-GMP Guanosine 3′:5′-cyclic monophosphate; AKH Adipokinetic hormone; PDE Phosphodiesterase.

RESULTS

Concentrations of c-AMP and c-GMP in the flight muscles of the locust at various times during a 30 min flight period are given in Fig. 1.

No significant changes in the content of either nucleotide could be demonstrated. The ratio of the c-AMP level to the level of c-GMP remained approximately 10. Injection of theophylline prior to the beginning of flight did not alter the concentrations of both nucleotides during a 30 min flight period.

Injections of octopamine, however, resulted in an increase of the c-AMP level. As indicated in Fig. 2, 5 min after injection of octopamine up to a haemolymph concentration of 0.4 μM, the level of c-AMP is raised from 10.4 $\pm$ 1.8 (saline injected locusts) to 17.6 $\pm$ 3.6 pmol/mg protein ($P < 0.01$). 10 min after injection the resting level is reached again.

A concentration of 400 μM octopamine results within 5 min after injection in a c-AMP level of 79.2 $\pm$ 13.9 pmol/mg protein ($P < 0.01$), this concentration remains high during the next 5 min. In both cases the c-GMP level was not influenced.

Injection of theophylline prior to the injection of octopamine did not enhance the effect on the c-AMP level.

Figure 3 shows the effects of injection of corpora cardiaca extracts on the cyclic nucleotide levels in flight muscle. Neither c-AMP nor c-GMP showed any response after injection of either 0.01 or 0.1 corpus cardiacum equivalents.

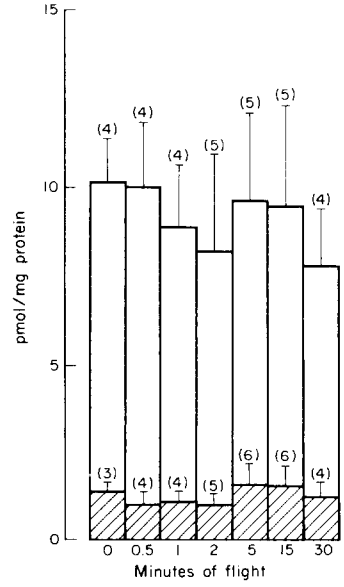

Fig. 1. Time-course of the changes in cyclic nucleotide contents of locust flight muscle during flight. For each experiment mean value and standard deviation of a number of groups (given in parenthesis) of five individuals are given. Blank columns, cyclic AMP: hatched columns, cyclic GMP.

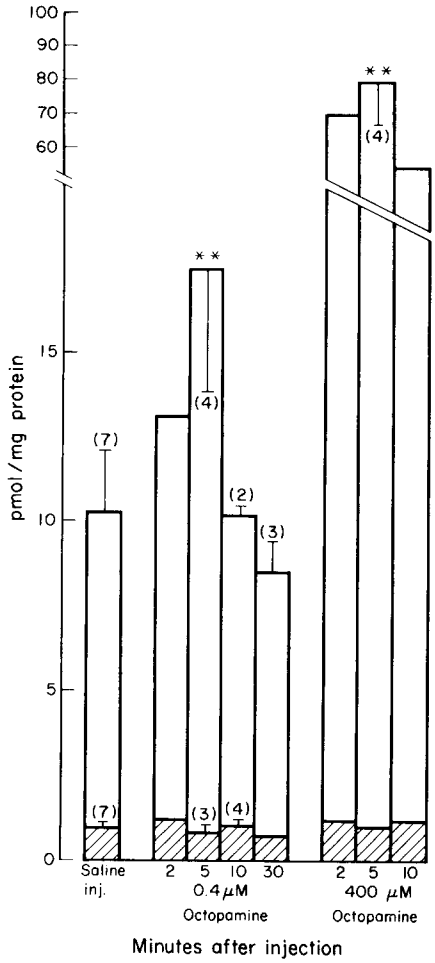

Fig. 2. Time-course of the changes in cyclic nucleotide contents of locust flight muscle after injection of octopamine in insect saline. Control groups received only saline injection. For each experiment mean value and standard deviation of a number of groups (in parenthesis) of five individuals are given. Blank columns, cyclic AMP: hatched columns, cyclic GMP: $^{**}P < 0.01$.

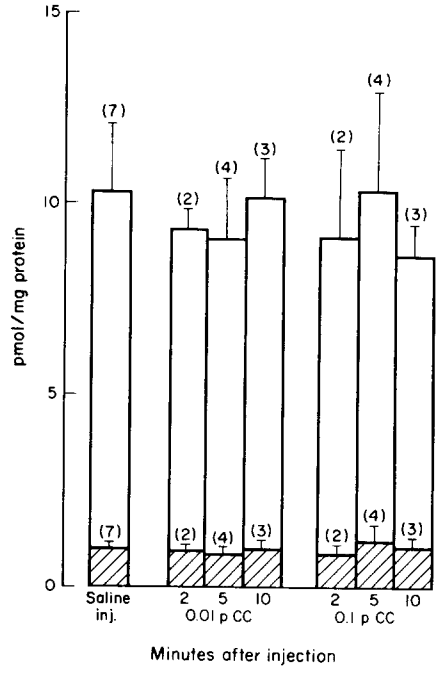

Fig. 3. Time-course of the changes in cyclic nucleotide contents of locust flight muscle after injection of an extract of corpora cardiaca. Control groups received only saline injection. For each experiment mean value and standard deviation of a number of groups (in parenthesis) of five individuals are given. Blank columns, cyclic AMP: hatched columns, cyclic GMP.

Injection of A23187 (final concentration 4 nM) dissolved in insect saline resulted in a decrease of the c-AMP level to 8.0 ± 0.5 pmol/mg protein after 5 min but this effect is of low significance ($P < 0.05$) (Fig. 4). No significant effect on the c-GMP concentration was found.

A great difference was found in the c-AMP levels in the various haemolymph samples with a mean value of 60.6 ± 38.7 (n = 25) and a range of 11–116 pmol/ml haemolymph. This was also true for the c-GMP level with a mean value of 37.8 ± 22.4 (n = 25) and a range of 11–98 pmol/ml haemolymph. A high level of c-AMP in the flight muscle was not concomitant with a higher level in the haemolymph, both at rest and after the various injections.

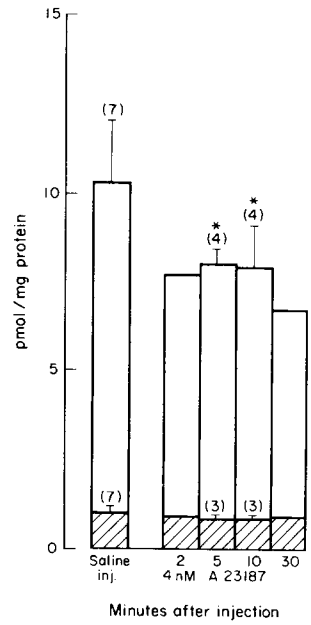

Fig. 4. Time-course of the changes in cyclic nucleotide contents of locust flight muscle after injection of A23187 in insect saline. Control groups received only saline injection. For each experiment mean value and standard deviation of a number of groups (in parenthesis) of five individuals are given. Blank columns, cyclic AMP: hatched columns, cyclic GMP. $^{*}P < 0.05$.

DISCUSSION

During the first 30 min of flight trehalose is the main substrate as witnessed by the fact that its haemolymph level decreases to about half the initial con-

centration (Robinson & Goldsworthy, 1976). Concomitant with this decrease the diglyceride content of the haemolymph is raised at the expense of fat body lipids under the action of the adipokinetic hormone (Beenakkers, 1969; Mayer & Candy, 1969), but it is uncertain as to how this is done.

It is known that extracts of corpora cardiaca of the cockroach, *Periplaneta americana*, contain a hyperglycaemic factor which raises c-AMP concentration (Hanaoka & Takahashi, 1977) and activates phosphorylase in the fat body (Steele, 1963). Injection of corpus cardiacum extract in *Locusta migratoria* evokes in the fat body an increase in c-AMP level (Gäde & Holwerda, 1976), an activation of a protein kinase as well as a stimulation of phosphorylase activity (Beenakkers *et al.*, 1978). In nerve cord of *Periplaneta americana* phosphorylase activity can be raised not only by corpora cardiaca extract but also by c-AMP or by the PDE inhibitor caffeine (Hart & Steele, 1973).

Robinson & Goldsworthy (1977) found that corpora cardiaca extract stimulated lipid utilization in the flight muscle of *Schistocerca gregaria*, together with a reduction of the glycolytic flux; the process involved is still unclear. Participation of c-AMP or c-GMP seems to be excluded, as in the present paper it is shown that corpus cardiacum extract does not alter the concentrations of these nucleotides in locust flight muscles. Candy (1978) however, finding no influence of corpora cardiaca extract on trehalose oxidation, demonstrated a marked stimulation of the oxidation of glucose and trehalose caused by octopamine. Although David & Lafon-Cazal (1979) recently reported rather high amounts of octopamine in locust corpora cardiaca, injection of 0.1 pair corpora cardiaca equivalents would result in a haemolymph concentration of femtomole order, whereas all reported effects of octopamine demand concentrations of at least picomole order. Candy (1978) showed that besides octopamine, both dibutyryl-c-AMP and theophylline stimulated the oxidation of glucose and trehalose and these data suggest that octopamine exerts its effect by raising the cyclic AMP level. Indeed, our experiments show that octopamine enhances the c-AMP level but not c-GMP level. Although haemolymph nucleotide levels vary to a great extent it must be emphasized that even when 20% of the liquid volume of the flight muscle is taken by intracellular space (Ford & Candy, 1972) their concentrations would only contribute to the flight muscle extract in negligible amounts. Because octopamine does not effect the PDE of the thoracic ganglia of *Periplaneta americana* it is supposed that the octopamine induced increase of c-AMP in this tissue is due to stimulation of adenylate cyclase and not to inhibition of PDE (Nathanson & Greengard, 1973). The decreasing effect of the calcium ionophore A23187 on c-AMP level reported in this paper, however, most likely acts through an enhanced activity of PDE, rather than through effect on adenylate cyclase. The fact that the PDE inhibitor theophylline did not enhance any of the effects reported, might be a result of a too short pre-incubation time prior to the injection of octopamine or corpora cardiaca extract.

As reported in the present paper c-AMP level in the flight muscles is raised by octopamine. One of the possibilities would be that this raise in c-AMP level acts on flight muscle glycogen phosphorylase. In nerve cord of *Periplaneta americana* octopamine had a pronounced glycogenolytic effect mediated by c-AMP (Robertson & Steele, 1972) and acting through activation of glycogen phosphorylase. In the locust flight muscle, however, this effect would be of importance only shortly after the onset of flight. As reported previously (Worm & Beenakkers, 1979) flight muscle glycogen decreases within 5 min of flight to reach a constant level of 16 μmol/g fresh weight, so flight muscle glycogen stores are not fully depleted. This could be the result of inhibition of glycogen phosphorylase by glucose-6-phosphate, as was shown to occur in the fat body of *Locusta migratoria* (Applebaum & Schlesinger, 1973). We found an increase of glucose-6-phosphate in the flight muscle shortly after the beginning of flight (Worm & Beenakkers, 1979).

The fact that c-AMP reaches its maximum 5 min after the administration of octopamine, while glycogen breakdown already has been terminated by that time is not contradictory to the view that octopamine exerts a c-AMP mediated role in flight muscle metabolism. It has been shown recently that total c-AMP is not a linear indicator of protein kinase activation, in contrast to protein bound c-AMP. Protein kinase activation was reported to be fully maximal within 2–5 min, whereas total c-AMP concentration still increases (Schumacher & Hilz, 1978). The capacity of fully stimulated cells to raise c-AMP level appears to be far in excess of what is needed for complete activation of protein kinase.

Our results indicate a c-AMP mediated role of octopamine in flight muscle metabolism acting by activation of glycogen phosphorylase, thus enabling increased glycolytic flux at the onset of flight. The way in which corpora cardiaca hormones might influence metabolism in favour of fatty acid oxidation remains still unclear.

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