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Developmental Biology

MECHANISM OF PUPARIATION DELAY INDUCED BY CAFFEINE IN *MUSCA DOMESTICA*

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The effect of cyclic nucleotides alone and in combination with caffeine on pupariation of *Musca domestica* was checked. Cyclic nucleotides when given alone to the larvae, caused an acceleration of pupariation. The accelerating effect observed was dependent on the age of the larvae. Dibutyryl cAMP seemed to show maximum effect compared to cAMP and cGMP. In combination treatment with caffeine, cAMP reversed caffeine-induced pupariation delay.

INTRODUCTION

Our earlier studies have shown that caffeine interferes with hormonally mediated responses in *Musca domestica* (Srinivasan & Kesavan, 1977, 1978). Similar observations have also been made on other insects (Blaustein & Schneiderman, 1960; McDaniel & Berry, 1974; Sehgal *et al.*, 1977). However, the mechanism by which caffeine brings about these effects remains unknown.

Caffeine is known for its pleotropic effects at the cellular level (Srinivasan, 1977). The established effects are : release of membrane-bound calcium and disruption of membrane function (Weber, 1968; Paul & Goff, 1973; Acosta & Anuforo, 1976), inhibition of macromolecular syntheses and inhibition of a variety of enzymes (Kihlman, 1977; Timson, 1977). The demonstration that caffeine is a potent inhibitor of cAMP phosphodiesterase (Butcher & Sutherland, 1962) makes it plausible to assume that accumulation of cAMP may have a role in the observed caffeine effects *in vivo*. Cyclic nucleotides have been implicated in the action of diverse insect hormones (Applebaum & Gilbert, 1972; Prince *et al.*, 1972; Vandenberg & Mills, 1974; Bodnaryk, 1975; Rasenick *et al.*, 1976; Everson & Feir, 1976; Achazi *et al.*, 1977).

This study was undertaken in order to test the above hypothesis. This report demonstrates that exogenously added cAMP accelerates pupariation and also reduces the magnitude of damage induced by caffeine.

MATERIALS AND METHODS

The strain of houseflies used in the present study (*Musca domestica* nebulo Fabr.) has been maintained in our laboratory at 31 ± 1°C. Techniques used for rearing the houseflies, collection and incubation of eggs have been the same as described earlier (Srinivasan & Kesavan, 1977).

The experiments were performed on 28, 38 and 50 hr old larvae. Caffeine and cyclic nucleotides of desired concentrations were prepared in milk. Fifty larvae were seeded in a beaker of 250 ml capacity and three replicates were prepared for each treatment. The experiments were carried out at 35±1°C, as this temperature supports

maximum rate of development. Scoring the puparia formed and the method used to determine pupariation, delay/acceleration were according to Srinivasan & Kesavan (1978). cAMP, dibutyryl cAMP, cGMP, papaverine and caffeine were obtained from Sigma Co., USA.

Results

Cyclic nucleotides influence pupariation markedly. Table I shows the extent of pupariation acceleration exerted by different cyclic nucleotides. Acceleration is maximum in 38 hr old larvae compared to other larval ages. Of the nucleotides employed here, dibutyryl cAMP exerts maximum acceleration followed by cAMP and cGMP.

TABLE I

Effect of cyclic nucleotides on pupariation of Musca domestica *larvae*

Age of the larvae (Hours)		Concentration of cyclic nucleotide in milk (μg/ml)	Acceleration of * pupariation (Hours)
28	cAMP	50	5·5
		100	6·5
	dbcAMP	50	6·0
		100	7·0
38	cAMP	50	8·0
		100	8·5
	dbcAMP	50	8·0
		100	10·0
	cGMP	50	7·5
		100	7·5
50	cAMP	50	3·5
		100	4·0
	dbcAMP	50	5·0
		100	5·0
	cGMP	50	3·0
		100	2·5

* Acceleration was determined graphically by comparing the time taken for 50% pupariation in cyclic nucleotide treated and untreated larvae. Each datum is based upon three replicas of 50 larvae each.

Caffeine effect on pupariation was checked by giving exogenous cyclic nucleotides. The results are presented in Table II. Pupariation is greatly affected as 28 hr old larvae show 15 and 62 hr delay and 38 hr old larvae show only 5 and 12 hr delay at 0.05 % and 0.1 % caffeine treatment respectively. It is obvious from the data that exogenously added cyclic nucleotides in combination treatment with caffeine fail to restore the damage induced by caffeine in 28 hr old larvae. However, 38 hr old larvae show reversal of caffeine-induced delay by cyclic nucleotides.

TABLE II

Influence of cyclic nucleotides on pupariation delay induced by caffeine in Musca domestica *larvae*

Age of the larvae (Hours)	Concentration of Caffeine (%)		Concentration of cyclic nucleotide (μg/ml)	Percentage* reversal of delay
28	0·05	cAMP	50	0
			100	5
		dbcAMP	50	5
			100	10
	0·1	cAMP	50	0
			100	0
		dbcAMP	50	0
			100	0
38	0·05	cAMP	50	70
			100	80
		dbcAMP	50	80
			100	90
	0·1	cAMP	50	20
			100	40
		dbcAMP	50	35
			100	50

* Percentage reversal of delay was calculated by comparing the time taken for 50% pupariation in caffeine and caffeine+cyclic nucleotide treated larvae. Each datum is based upon three replicas of 50 larvae each.

Papaverine, an inhibitor of cAMP phosphodiesterase, induces a delay in pupariation similar to methyl xanthines at higher concentrations (Table III).

TABLE III

Effect of papaverine on pupariation of Musca domestica *larvae**

Concentration of papaverine (%)	Pupariation** delay (Hours)
0·05	9
0·1	14
0·2	14

* 38 hr old larvae were used.

** Each datum is based upon three replicas of 50 larvae each.

DISCUSSION

Several criteria must be fulfilled before cAMP can be implicated as a mediator in the mechanism of action of any given substance. As outlined by Robison *et al.* (1971),

the effects of drug or chemical agent should be mimicked by exogenous cAMP. Addition of cAMP in the medium produced a significant acceleration of pupariation in *Musca domestica* contrary to the observations made with caffeine. Acceleration of pupariation has also been observed in insect larvae treated with venom of scorpion, ecdysone and electrical stimulation (Robbins *et al.*, 1968; Thomson & Horn, 1969; Fraenkel & Zlotkin, 1970).

Pupariation in flies is under neuroendocrine influence: the neurosecretory system in the brain reacting to stimuli arising during maturation process, releases the prothoracotrophic hormone to activate the ring gland (RG) which then secretes the molting hormone, ecdysone. The dramatic rise in ecdysone titre before pupariation has been noted in a number of insects (Slama *et al.*, 1974). There is a growing body of evidence which suggests the role of an adenyl cyclase system in the mechanism of steroid hormone action in insects (Applebaum & Gilbert, 1972; Vedeckis & Gilbert, 1973; Bodnaryk, 1975; Rasenick *et al.*, 1976) and both adenyl cyclase and cAMP phosphodiesterase activity have been found to exhibit changes during insect development (Castillon *et al.*, 1973; Catalan *et al.*, 1975). De Reggi & Cailla (1975) have found a sharp increase in cAMP content before puparium formation in the larvae of *Drosophila melanogaster*. On the basis of these studies, it can be envisaged that in *Musca domestica* larvae the added exogenous cAMP may elevate its level in the respective organs well before the schedule and may influence pupariation.

cGMP also promotes pupariation (Table I). The nature of the interaction between cGMP and the hormones involved in pupariation are unknown at present. It is worth mentioning that the high levels of cyclic GMP (Kuo *et al.*, 1972; Fallon & Wyatt, 1975; Hayes *et al.*, 1976) and the apparent predominance of cyclic GMP-dependent protein kinases (Kuo *et al.*, 1971) in some insect tissues may be important in biochemical regulation in insects.

The rate of pupariation acceleration caused by cAMP is dependent on the age of the test larvae (Table I) and seems to be different in this regard from that of scorpion venom or electrical stimulation. This suggests that the stimulatory effects of cAMP may be due to its effect on prothoracic glands to release ecdysone. The puparia obtained from the larvae treated with cyclic nucleotides were normal in shape as against malformed puparia induced by electrical stimulation and scorpion venom (Fraenkel & Zlotkin, 1970).

Caffeine delayed pupariation and the delay is further dependent on the age of larvae. Considering cAMP phosphodiesterase inhibition by methyl xanthines, one should expect similar results both with exogenous cAMP and caffeine. It may be suggested that caffeine may not be able to elevate the intracellular cAMP in housefly larvae by blocking its degradation as expected. Such a situation has been shown to exist in different systems (Chang, 1968; Pastan & Perlman, 1970). Further, McDaniel (1973) observed nonsignificant increase in cAMP in caffeine-treated moth *Hyalophora cecropia*. Hayes *et al.* (1976) observed similar results in tobacco bud worm after administering caffeine to the larvae. Strikingly, papaverine also delays pupariation. Hence, one might argue that the delay in pupariation by caffeine and

papaverine could be due to their effect on other target sites (Browning *et al.*, 1974; Srinivasan & Kesavan, 1978).

The studies of McDaniel *et al.* (1976) and Johnson *et al.* (1976) on methyl xanthine-arrested *Hyalophora cecropia* have shown that differentiation of prothoracic glands is blocked. Our results concerning the reversal of caffeine-induced delay by cyclic nucleotides may be due to the activation of the caffeine-inhibited prothoracic glands. Indeed, Vedeckis *et al.* (1974) have reported certain connections between the action of brain hormone, adenyl cyclase activity and the formation of ecdysone in the prothoracic gland. Further studies, mainly determination of cAMP content in housefly larvae, are in progress to explain this phenomenon.

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