Involvement of Cyclic Adenosine Monophosphate in the Regulation of Pupal Color Adaptation of the Butterfly, *Inachis io*

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In the butterfly *Inachis io*, a pupal melanization reducing factor (PMRF) which is located throughout the entire central nervous system controls the intensity of pigmentation of pupal cuticle depending on the background color of the pupation site. PMRF does not only reduce melanization but, in addition, enhances lutein incorporation in a dose-dependent manner to form pupae with yellow color on bright backgrounds.

The present paper reports on the effects on pupal pigmentation caused by cyclic nucleotides and phosphodiesterase (PDE) inhibitors which prevent degradation of cyclic nucleotides. The injection of cAMP did not alter pupal coloration whereas its membrane-permeable analog dibutyryl-cAMP mimicked dose-dependently PMRF activity. Thus, pupae of reduced melanization and, in addition, enhanced yellow coloration were formed. This indicates that an increased intracellular cAMP level is capable of mediating PMRF effect. Also, the injection of the PDE inhibitor isobutylmethylxanthine (IBMX) caused dose-dependently pupae of reduced melanization and enhanced lutein incorporation.

Theophylline (another PDE inhibitor) was only slightly effective (23% inhibition of melanization) at the highest dose compared to IBMX. The injection of cGMP and its analog dibutyryl-cGMP exhibited no melanization reducing effect.

Extracts of abdominal ganglia (AG) which contained PMRF activity caused significantly brighter pupae when injected in combination with IBMX. However, this stimulation by IBMX became no longer effective at higher AG doses. Therefore, the present results are suggestive of an involvement of cAMP as a second messenger in the action of PMRF on pupal color adaptation.

Introduction

Some rhopaloceran butterfly pupae which are not covered by cocoons or buried in the ground show a cuticular pigmentation which depends on the background color of the pupation site (Koch and Bückmann, 1984). This morphological color adaptation is controlled by a hormonal factor which reduces melanization and, therefore, has been called pupal melanization reducing factor (PMRF) (Bückmann, 1960; Bückmann and Maisch, 1987). In the peacock butterfly *Inachis io* L. (Lepidoptera: Nymphalidae), PMRF does not only affect melanization but, in addition, seems to be responsible for enhancing lutein incorporation into cuticle (Starnecker, 1997). PMRF is a peptide as demonstrated by enzymatic digestion (Bückmann and Maisch, 1987) and located throughout the entire prepupal central nervous system (CNS) (Starnecker *et al.*, 1994; Starnecker, 1996a), but its release in those prepupae which are adapted to a yellow background (Starnecker and Bückmann, 1997) is controlled by nervous stimulus from the brain as was shown by nerve severance experiments (Bückmann, 1969, 1971). PMRF is not only present in prepupal CNS when it is urgently required because wandering larvae seem to prefer light environments for pupation (Starnecker, 1996b), but occurs also in first instar larvae as well as in adults (Starnecker and Bückmann, 1997). However, its function in these developmental stages is yet unknown.

The role of cyclic nucleotides in insects in mediating hormone action as second messenger was shown in a series of reports (see Smith and Combest, 1985). In a first study it is examined whether the injection of cyclic nucleotides, their analogs, and the cyclic nucleotide phosphodiesterase inhibitors isobutylmethylxanthine (IBMX) and theophylline exhibit an effect on the hormonally

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controlled morphological color adaptation of *I. io* pupae.

Materials and Methods

Animals

Inachis io was reared in a permanent stock colony (Maisch and Bückmann, 1987; Starnecker *et al.*, 1994). Last instar larvae were kept in boxes (30x35x35 cm) which are covered with a dome of gauze (height 30 cm). Wandering larvae leave stinging nettle (*Urtica dioica*) and move up to this dome which is now partly covered black. Such animals were collected either for the bioassay or for dissection of abdominal ganglia (AG) to extract PMRF.

Dissection of ganglia and PMRF extraction

400 AG complexes comprising 7 separate ganglia were dissected in a lepidopteran Ringer as described previously (Starnecker *et al.*, 1994) and homogenized by sonication (Branson sonifier W250). The extraction was performed according to Starnecker (1996a) with 80% ethanol in water (v/v) and 2M acetic acid three times each. Ethanol was removed by lyophilization prior to a purification step on solid phase RP-C18 cartridges. The cartridges were rinsed with 25 and 75% acetonitrile in water (v/v) containing 0.1% trifluoroacetic acid (Starnecker and Bückmann, 1997). The latter solution containing PMRF activity was lyophilized and redissolved in water to give the highest dose of 16 AG complex equivalents per 10 µl.

Bioassay for PMRF activity

The bioassay was performed according to Maisch and Bückmann (1987). Two-hour old prepupae which were adapted as wandering larvae to a black background and normally develop into strongly melanized pupae were injected with 10 µl of test solution. The degree of melanization of the resulting pupae is determined by a scoring system of 5 classes, where class 5 represents the most and class 1 the least intense melanization (Maisch and Bückmann, 1987). A mean melanization score of 1 corresponds to a 100% reduction of pupal melanization and indicates strong PMRF activity.

Chemicals

The cyclic nucleotides 3',5'-cAMP, 3',5'-cGMP, their analogs dibutyryl-3',5'-cAMP, dibutyryl-3',5'-cGMP, and the phosphodiesterase (PDE) inhibitors isobutylmethylxanthine (IBMX) and theophylline (=dimethylxanthine) were purchased from Sigma. They were dissolved in water at increasing doses per 10 µl and tested in 2-h old prepupae (see above) for their effect on pupal melanization.

Statistical significance was calculated using Wilcoxon's rank test.

Results and Discussion

The injection of cAMP at doses between 0.1 and 3 µmol into prepupae adapted to a black background did not cause a reduction of melanization, yielding pupae of melanization scores similar to pupae treated with water (Fig. 1). When increasing doses of dibutyryl-cAMP were injected, melanization of pupae was reduced in a dose-responsive manner (Fig. 1). Additionally, all pupae showed an intense yellow coloration indicating that also the inversely related pigmentation was affected. One µmol of dibutyryl-cAMP was the lowest dose that is capable to mimic significantly PMRF activity. The results indicate that cAMP cannot penetrate the plasma membrane of the target tissue whereas the injection of dibutyryl-cAMP , a lipid-soluble

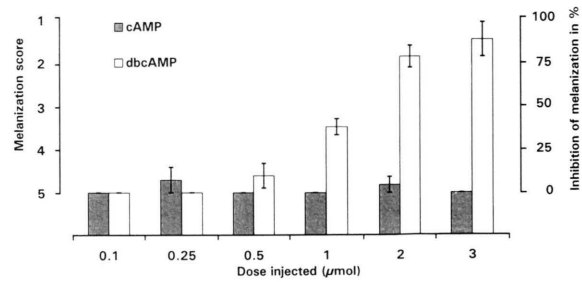

Fig. 1. Effects of cyclic adenosine monophosphate (cAMP) and its analog dibutyryl-cAMP (dbcAMP) on pupal melanization after injection at different doses per 10 µl into 2-h old prepupae adapted to a black background. The intensity of melanization is expressed in melanization scores (MS) (see Materials & Methods) and the corresponding percentage of melanization inhibiting effect. Each bar represents the mean ± SEM for 6 animals. Controls: untreated animals adapted to a black background, MS > 4.5; untreated animals adapted to a yellow background, MS < 1.5; animals adapted to a black background and injected with 10 µl water, MS = 4.9 ± 0.06).

cAMP analog, enhances intracellular cAMP to initiate both pigmentation effects. Dibutyryl-cAMP was shown to mimic also the effect of adipokinetic hormone in *Locusta migratoria* (Gäde and Holwerda, 1976), the trehalosemic effect of corpora cardiaca extracts (Hanaoka and Takahashi, 1977), the tanning effect of bursicon (Vandenberg and Mills, 1974) in *Periplaneta americana*, the steroidogenic effect of the prothoracicotropic hormone in *Manduca sexta* (Smith *et al.*, 1984; Watson *et al.*, 1993) or the pheromone production effect of the pheromone biosynthesis activating neuropeptide in *Bombyx mori* (Fonagy *et al.*, 1992). An involvement of cGMP in mediating the effect of the eclosion hormone was reported for *Hyalophora cecropia* (Truman *et al.*, 1979). By contrast, neither cGMP nor its analog dibutyryl-cGMP showed a melanization reducing effect when tested in the *I.io* PMRF bioassay (data not shown).

IBMX and theophylline are known to inhibit PDE to degrade cAMP. The injection of IBMX, beginning at a dose of 25 nmol exhibited pupae of significantly decreased melanization (Fig. 2). At a dose of 100 nmol IBMX (higher quantities do not solve completely in water as it is the case for theophylline) pupae with a mean melanization score of 2.0 were formed. Theophylline which is generally less potent than IBMX (Smith and Combest, 1985), showed almost no effect. Only pupae which were injected with the highest dose of 100 nmol revealed a slight melanization reducing effect of 23% (Fig. 2). This is comparable to the ecdysone synthesis in the prothoracic glands of *M. sexta* (Smith *et al.*, 1984), and the pheromone production in pheromone glands of *Heliothis armigera* (Rafaeli and Soroker, 1989) which could be stimulated by IBMX incubations. Injection of IBMX into isolated abdomen of *H. cecropia* was about 100 times more effective in inducing eclosion behavior than theophylline (Truman *et al.*, 1979).

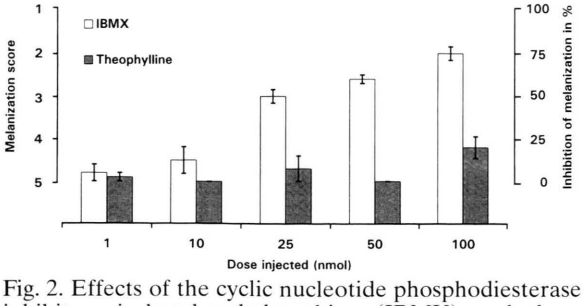

Fig. 2. Effects of the cyclic nucleotide phosphodiesterase inhibitors isobutylmethylxanthine (IBMX) and theophylline (=dimethylxanthine) on pupal melanization after injection at different doses per 10 µl into 2-h old prepupae adapted to a black background. Each bar represents the mean ± SEM for 6 animals. For further explanations and control values see Fig. 1.

In a further experiment, increasing doses of cAMP together with 10 nmol IBMX (the highest dose which alone did not alter pigmentation) were injected into prepupae. All resultant pupae did not show reduced melanization, revealing melanization scores of > 4.5. Also, in combination with higher doses of IBMX pupae exhibited only the scores which were affected by IBMX alone (data not shown). Thus, it is more likely that the offered cAMP could not penetrate plasma membrane than that it is rapidly degraded by PDE.

To see whether IBMX can sustain the effect of PMRF, increasing doses of AG extracts containing 10 nmol IBMX were injected into prepupae. At low AG doses betweeen 0.25 and 1 equivalents the presence of IBMX caused significantly brighter pupae in comparison to pupae which were treated with AG extracts only (Fig. 3). Thus, IBMX presumably synergized the PMRF effect by maintaining higher intracellular cAMP levels. However, this effect became no longer effective at higher AG doses. An enhancing of hormone effects caused by additionally applied IBMX was also reported for the eclosion hormone (Truman *et al.*, 1979), for the adipokinetic hormone (Orchard *et*

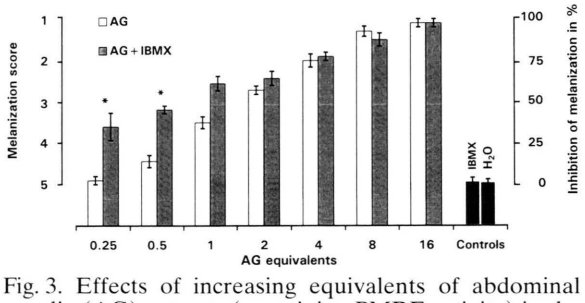

Fig. 3. Effects of increasing equivalents of abdominal ganglia (AG) extracts (containing PMRF activity) in the presence and absence of isobutylmethylxanthine (IBMX) on pupal melanization. The dose of 10 nmol IBMX per 10 µl is the highest dose which alone did not affect melanization reduction (see also Fig. 2). Each bar represents the mean ± SEM for 6–8 animals. Significance between corresponding doses of AG and AG + IMBX: *, $p<0.05$. Controls: animals injected either with 10 nmol IBMX in 10 µl of water or water alone. For further explanations see Fig. 1.

al., 1982), and the pheromonotropic hormone (Fonagy *et al.*, 1992). In the latter case the enhancing effect ceased at higher doses of the hormone which is similar to the present result on PMRF activity.

The criteria for the demonstration of cAMP as a second messenger according to E. Sutherland and his colleagues (see Smith and Combest, 1985, p. 271) is as follows:

(1) the hormone should specifically stimulate adenylate cyclase activity;

(2) the hormone should increase intracellular levels of cAMP in its target tissue;

(3) effects of the hormone should be potentiated by inhibitors of phosphodiesterase;

(4) effects of the hormone should be mimicked by exogenous cAMP or cAMP analogs.

The present results show that injected dibutyryl-cAMP and IBMX mimic the PMRF effect and IBMX enhances PMRF activity when injected in combination. Therefore, it is likely that cAMP is involved as a second messenger in the action of PMRF, although the increase of intracellular cAMP and the stimulation of adenylate cyclase of the target tissue affected by PMRF remain to be demonstrated in further investigations.

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