Occurrence of a Pupal Melanization Reducing Factor in Different Insects

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A pupal melanization reducing factor (PMRF) controls incorporation of pigments into the cuticle of pupae of the nymphalid butterfly, *Inachis io*, to adapt their coloration to the background colors of their pupation sites. In the present paper, we report on the presence of PMRF in the central nervous system (CNS) of further butterfly species which exhibit a pupal morphological color adaptation. Interestingly, two butterfly species which do not show such an adaptation, and even two moth species which pupate in cocoons also contained PMRF in their CNS. The ganglionic extracts of non-lepidopteran species did not show a PMRF activity. The occurrence of PMRF (or PMRF-like molecules), therefore, may be restricted to the lepidopteran order.

Introduction

Some rhopaloceran butterfly pupae adapt the color of their cuticle to the background color of the pupation site (Bückmann, 1974; Koch and Bückmann, 1984; Koch *et al*., 1990). In the peacock butterfly, *Inachis io*, this morphological color adaptation is controlled by a peptide hormone which reduces the cuticular incorporation of melanin in pupae and was named pupal melanization reducing factor (PMRF) (Bückmann and Maisch, 1987). PMRF is located throughout the entire central nervous system (CNS) of *I. io* (Starnecker *et al*., 1994a) and released during the onset of the prepupal stage (Starnecker and Bückmann, 1996). Consequently, PMRF is a hormone which is necessary only once in *I. io*'s life history during a short period of time.

Interestingly, PMRF has been extracted in one case each from the prepupal brain-suboesophageal ganglion complex of a butterfly which exhibits no pupal color adaptation (Starnecker *et al*., 1994b), from head-prothorax fragments of a moth (Koch *et al*., 1990), and even from sinus glands of a crustacean (Bückmann *et al*., 1990). Therefore, the occurrence of PMRF in different insects is examined on a larger scale.

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Materials and Methods

Animals

Larvae of the cabbage white, *Pieris brassicae*, were collected in the field and fed with cabbage. Larvae of the following species were reared in a permanent stock colony in our laboratory: the peacock butterfly, *I. io*, and the small tortoiseshell, *Aglais urticae*, were fed with stinging nettle, the African satyrid, *Bicyclus anynana*, with maize and the North American buckeye, *Precis coenia*, with a semiartificial diet containing dried plantain. Larvae of the painted lady butterfly, *Vanessa cardui*, were purchased from Carolina Biological Supply Company and fed a semiartificial diet with dried thistle leaves. Eggs from the tobacco hornworm, *Manduca sexta*, were a gift from Dr. J. Milde, Köln. Their larvae also were fed a semiartificial diet containing dried tobacco leaves. All larvae and adults were kept under long day conditions (18h L : 6h D) and at 23–25°C. The butterflies were fed a 10% honey solution. Ganglia complexes of the silkworm, *Bombyx mori*, were a gift from Dr. S. Matsumoto, Wako-shi, Saitama, Japan. The cricket, *Gryllus bimaculatus*, was reared in our laboratory on a mixture of rabbit and mouse diets. The cockroach, *Blaberus craniifer,* the African migratory locust, *Locusta migratoria,* and the neotropical tenebrionid beetle, *Zophobas atratus*, were from a commercial dealer. The taxonomic classification of the different lepidopteran species and the presence or absence of a morphological color adaptation within these species are listed in Table I. Animals of other insect orders are listed in Table II.

Extraction and bioassay

For PMRF extraction, the butterfly and moth larvae at the age between wandering phase and young prepupae were collected and stored frozen until dissection. *B. craniifer, G. bimaculatus* and *L. mi-*

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gratoria were dissected as last instar nymphs and adults, *Z. atratus* as last instar larvae. The CNS of 160 animals each were dissected and separated into 4 complexes consisting of brain-suboesophageal (Br-SOG), thoracic ganglia 1–3 (TG_{1-3}) and abdominal ganglia (AG) 1–4 and/or 5–7, respectively. In the case of non-lepidopteran species, the abdominal nerve chain remained together (AG_{1-7}). All samples were stored in ice-cold pure acetone and extracted with 80% ethanol in water (v/v) (Starnecker *et al.*, 1994a), followed by a re-extraction of the pellet with 2 M acetic acid which resulted in a higher yield of PMRF. In the case of *B. mori*, *M. sexta*, *B. craniifer*, *G. bimaculatus*, and *L. migratoria*, lipids were removed with n-hexane and diethylether prior to Sep-Pak purification (Starnecker and Bückmann, 1996). For the *I. io* bioassay (Bückmann and Maisch, 1987), all samples were lyophilized and redissolved in water to give the highest dose of 16 complex equivalents per 10 µl. Injections were carried out into 2 hour old prepupae of *I. io* kept on a black background which normally develop into strongly melanized pupae. A PMRF activity is shown by a reduced pupal melanization. The degrees of melanization were scored into 5 classes, where class 5 represents the most intense and class 1 the least intense melanization (Maisch and Bückmann, 1987).

Results and Discussion

In *I. io*, PMRF was present in all 4 parts of the CNS (Table I). The higher activities of PMRF in the posterior parts (AG_{1-4} and AG_{5-7}), revealing melanization scores (MS) of 1.6 and/or 1.8, compared to those in both anterior parts (Br-SOG; MS 2.7 and TG_{1-3}; MS 2.3) are in agreement with earlier results (Starnecker *et al.*, 1994a). After a subdivision of the abdominal nerve cord into three portions of 2 ganglia each and the separate terminal ganglion, it was shown that all these portions contained PMRF activity which speaks for an equal distribution in every single ganglion (Starnecker *et al.*, 1994a). In comparison with the effect of the own PMRF, the extracts of *P. brassicae* generally yielded higher MS (except Br-SOG) indicating lower PMRF activities, those of *A. urticae* similar MS and those of *V. cardui* always significant lower MS and, therefore, higher PMRF activities in the *I. io* bioassay (Table I). That means that these 3 species which all show a color adaptation possess the same or a PMRF-like molecule as it does *I. io*. Also, the distribution of the hormone throughout the entire CNS is similar to the results obtained from *I. io*. The lower and/or higher hormone activities may be due to different amounts of the hormone in the ganglia complexes or to a different potency of the PMRF homologues.

In pupae of *I. io*, it was demonstrated that PMRF may have a dual function causing dose-dependently both a reduction of cuticular melanization and a stimulation of lutein incorporation (Starnecker, 1996). Both effects also occur in *A. urticae* and *V. cardui* which formed metallic golden, yellow pupae when adapted to a light

Table I. PMRF activity of extracts from different parts of the nervous system from various lepidopteran families tested in *Inachis io* bioassay. Mean melanization score is given ± SEM for 5 animals each injected with 16 ganglia complex equivalents.

Family	Species	Pupae with color adaptation	Melanization score Br–SOG	TG 1–3	AG 1–4	AG 5–7
Pieridae	*Pieris brassicae*	yes	2.7 ± 0.26	3.2 ± 0.26	2.5 ± 0.27	2.6 ± 0.25
Nymphalidae	*Inachis io*	yes	2.7 ± 0.26	2.3 ± 0.20	1.6 ± 0.19	1.8 ± 0.12
	Aglais urticae	yes	2.3 ± 0.12	2.1 ± 0.10	1.7 ± 0.12	2.2 ± 0.12
	Vanessa cardui	yes	1.2 ± 0.10	1.2 ± 0.10	1.0 ± 0.00	1.1 ± 0.05
	Precis coenia	no	1.5 ± 0.22	1.8 ± 0.12	1.7 ± 0.26	2.4 ± 0.19
Satyridae	*Bicyclus anynana*	no	1.8 ± 0.12	3.0 ± 0.35	2.7 ± 0.12	2.9 ± 0.19
Bombycidae	*Bombyx mori*	no	2.3 ± 0.20	3.2 ± 0.26	1.0 ± 0.00	1.0 ± 0.00
Sphingidae	*Manduca sexta*	no	1.0 ± 0.00	1.0 ± 0.00	1.0 ± 0.00	1.0 ± 0.00

Parts of the central nervous chain: Br–SOG, brain-suboesophageal ganglion complex; TG 1–3, thoracic ganglia 1–3; AG 1–4, AG 5–7, abdominal ganglia 1–4, and 5–7. For control values see Table II.

background or treated with PMRF (Koch *et al.*, 1990). In *P. brassicae*, in addition to higher amounts of lutein in pupal integument, light pupae contain more bile pigments than the black ones (Kayser, 1974) to appear green. Whether PMRF is responsible for an enhancement of bile pigments and, therefore, may possess a further function has to be demonstrated. It only has been shown that PMRF extracts from *I. io* cause a reduction of melanization in pupae of *P. brassicae* (Koch *et al.*, 1990).

Although, the butterflies *P. coenia* and *B. anynana* show no pupal color adapation, both species contained PMRF activity in considerable amounts in all 4 CNS portions (Table I). Also, the moth species *B. mori* and *M. sexta* which pupate in cocoons possessed high PMRF activity in the CNS. Their AG extracts, above all, caused MS of 1.0 (Table I). PMRF activity could be demonstrated as well in head-prothorax extracts of the wax moth, *Galleria mellonella* (Pyralidae) (Koch *et al.*, 1990) and, therefore, is present in a further lepidopteran family. However, in the 4 species of the insect orders *Blattodea*, *Caelifera*, *Ensifera*, and *Coleoptera* no PMRF activity was detectable (at least in the developmental stages investigated) (Table II). Whether PMRF is present in other arthropod groups, in addition to its demonstration in crustacean sinus glands (Bückmann *et al.*, 1990), requires further investigations. For the present, the results speak in favour of an occurrence of PMRF (or PMRF-like molecules) among *Lepidoptera* only. It is assumed that the hormone has gained other functions, still unknown, in the lepidopteran species without color adaptation.

In *I. io*, PMRF is present in all developmental stages even in first instar larvae and adults (Starnecker and Bückmann, 1996) and, consequently, long before and after it is necessary for color adaptation during prepupal stage. Therefore, it was suggested that PMRF may serve other functions in these developmental stages. For that reason, its occurrence in locusts, cockroaches or beetles was not excluded. The occurrence of PMRF activity in the sinus glands of *Homarus americanus* (Bückmann *et al.*, 1990) is quite conceivable. For example, the adipokinetic hormones serving different functions in insects show sequence similarities and cross-reactivity with the red pigment concentrating hormone of crustaceans (Goldsworthy *et al.*, 1986; Gäde, 1990).

It is more likely that the melanization reducing effect caused by the extracts of various lepidopteran species in *I. io* is affected by different, but structurally related PMRF molecules all belonging to the same peptide family than by a single, identical one. A further example for a hormone with multiple functions is the pheromone biosynthesis activating neuropeptide (PBAN) which shares sequence similarities with the melanization and reddish coloration hormone (MRCH), the *B. mori* diapause hormone, and the leucopyrokinins. They all show cross-reactivity in the other bioassays (Nijhout, 1994). However, MRCH responsible for

Order: Family	Species	Melanization score Br–SOG	TG 1–3	AG 1–7
Blattodea: Blaberidae	*Blaberus craniifer*	4.7 ± 0.12	4.8 ± 0.20	5.0 ± 0.00
Caelifera: Acrididae	*Locusta migratoria*	4.2 ± 0.27	4.6 ± 0.40	4.5 ± 0.16
Ensifera: Gryllidae	*Gryllus bimaculatus*	4.6 ± 0.25	4.5 ± 0.39	4.8 ± 0.12
Coleoptera: Tenebrionidae	*Zophobas atratus*	4.9 ± 0.10	5.0 ± 0.00	5.0 ± 0.00
Controls:				
water injected animals on a black background		4.6 ± 0.11		
untreated animals on a black background		4.9 ± 0.07		
untreated animals on a yellow background		1.1 ± 0.07		

Table II. PMRF activity of extracts from different parts of the nervous system from animals of various insect orders tested in *Inachis io* bioassay. Mean melanization score is given ± SEM for 5 animals each injected with 16 ganglia complex equivalents.

Parts of the central nervous chain: Br–SOG, brain-suboesophageal ganglion complex; TG 1–3, thoracic ganglia 1–3; AG 1–7, all 7 abdominal ganglia.

the pigmentation of armyworm larvae shows no effect in *I. io* bioassay and therefore, MRCH and PMRF may be different molecules belonging to different peptide families (Starnecker *et al.*, 1994a). Nevertheless, *I. io* contains a MRCH/PBAN-like factor in its CNS which is active in the *B. mori* pheromone gland bioassay (Starnecker *et al.*, 1994a).

The occurrence of PMRF in all developmental stages of *I. io* (Starnecker and Bückmann, 1996) speaks for a hormone with multiple functions within a single species. Moreover, its occurrence in species of different lepidopteran families which do not exhibit a color adaptation suggests that PMRF is a polytropic hormone. Nevertheless, among insects PMRF seems to be restricted to the lepidopteran order where at least the amino acid sequence essential for PMRF activity is conserved. But a conservation of the amino acid sequence is not necessarily accompanied by a conservation of function (Duve and Thorpe, 1994) which is demonstrated by the presence of PMRF in butterflies without color adaptation and even in moths.

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