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Presence of [His^7]- corazonin in the central nervous system of a newly isolated albino strain of *Schistocerca gregaria* (Orthoptera: Acrididae) – mass spectrometric and immunocytochemical evidence

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Abstract. An inbred strain of a newly isolated spontaneous albino mutant of *Schistocerca gregaria* (Forsk.) was examined for the presence of the neuropeptide [His^7]-corazonin by immunocytochemical and mass spectrometric methods. It was concluded that this peptide is definitely present in a limited number of neurosecretory cells in the pars lateralis as well as in the corpora cardiaca (CC). Injection of either synthetic [His^7]-corazonin or of extracts of CC of the normal coloured phenotype of *S. gregaria* failed to induce darkening of the cuticle, while albino *Locusta migratoria*, used as a positive control, turned dark. The conclusion is that the cause of albinism in the new *S. gregaria* albino is probably due to a defect in the receptor system for [His^7]-corazonin or in the biosynthetic pathway of melanin.

INTRODUCTION

Body color changes occur in many insects in response to environmental cues such as population density, humidity, background color, temperature, photoperiod and food (Chapman, 1998). Locusts, in particular *Schistocerca gregaria* and *Locusta migratoria*, exhibit a wide range of color variation in response to population density. Nymphs reared at low density (solitarious form) are greenish, while those at high density (gregarious form) have black patterns. This color variation, being one of the most conspicuous phase-related phenomena, has been studied extensively (Faure, 1932; Uvarov, 1966; Fuzeau-Braesch, 1985; Pener, 1991; Pener & Yerushalmi, 1998).

To date, two hormones have been implicated in such phase-related color changes. Juvenile hormone promotes the greenish color, but, according to Pener & Yerushalmi (1998), it is not the key inducer of solitarization. The other hormone is [His^7]-corazonin, also described as "locust dark-color inducing neuropeptide" (Lom-DCIN, Tanaka & Pener, 1994) and "dark pigmentotropin" (Tawfik et al., 1999). It promotes the dark body color typical for the gregarious phase. This hormone can be identified by using an albino mutant of the Okinawa strain of *L. migratoria* for bioassay. This mutant turns black (after the next molt) upon implantation of corpora cadiaca (CC) from normal locusts, as well as upon injection of a methanolic extract of CC. This formed the basis for the chromatographic purification and the elucidation of the amino acid sequence of [His^7]-corazonin. The peptide has been also isolated from *S. gregaria*, and identified using the same *L. migratoria* albino strain for bioassay (Tawfik et al., 1999).

Recent results indicate that [His^7]-corazonin does not only affect the coloration, but also locust phase-dependant characteristics such as morphometrics and behaviour (Hoste et al., 2002a, b). We are therefore searching for suitable models, in particular in *S. gregaria*; hence our interest in albinos. During mass rearing of the desert locust, *S. gregaria* at our Institute, several albino individuals, creamy-white in color, were observed among the heavily pigmented ones, and an albino strain was established from them. If it were deficient in [His^7]-corazonin, it would offer the same advantages as the albino Okinawa strain of *L. migratoria*. This strain has now been bred over several generations without the appearance of any hoppers of the normal colored phenotype. Even under crowded rearing conditions, the albino hoppers do not develop the black markings as normal ones do. Albinism in established albino mutants of *L. migratoria* and *S. gregaria* is caused by a single recessive Mendelian unit (Hasegawa & Tanaka, 1994).

Several hypotheses have been put forward regarding albinism, such as (a) the precursor gene is mutated, (b) [His^7]-corazonin is not cleaved properly from its precursor, (c) the mutant lacks the releasing factors for [His^7]-corazonin, (d) the albinism is caused by a mutation in the [His^7]-corazonin receptor or in the signal transduction cascade, or (e) [His^7]-corazonin is a releasing factor for the dark color inducing hormone from the CC (Schoofs et al., 2000).

In this paper we report on how we reached the conclusion that in this particular albino strain of *S. gregaria*, the albinism is not due to any defect of the corazonin synthesis and release cascade.

MATERIALS AND METHODS

Experimental insects

Normally colored and albino strains of both *S. gregaria* and *L. migratoria* were reared under crowded conditions at 32 ± 1°C and a 13:11 h LD photo regime according to Ashby (1972). Cabbage leaves, grass and oat flakes were supplied as food.

Antisera production and immunocytochemistry

Synthetic [His^7]-corazonin (pGlu-Thr-Phe-Gln-Tyr-Ser-His-Gly-Trp-Thr-Asn-amide) was coupled to bovine thyroglobulin using glutaraldehyde as a cross-linker, and antisera were raised in rabbits as described by Baggerman et al. (2001).

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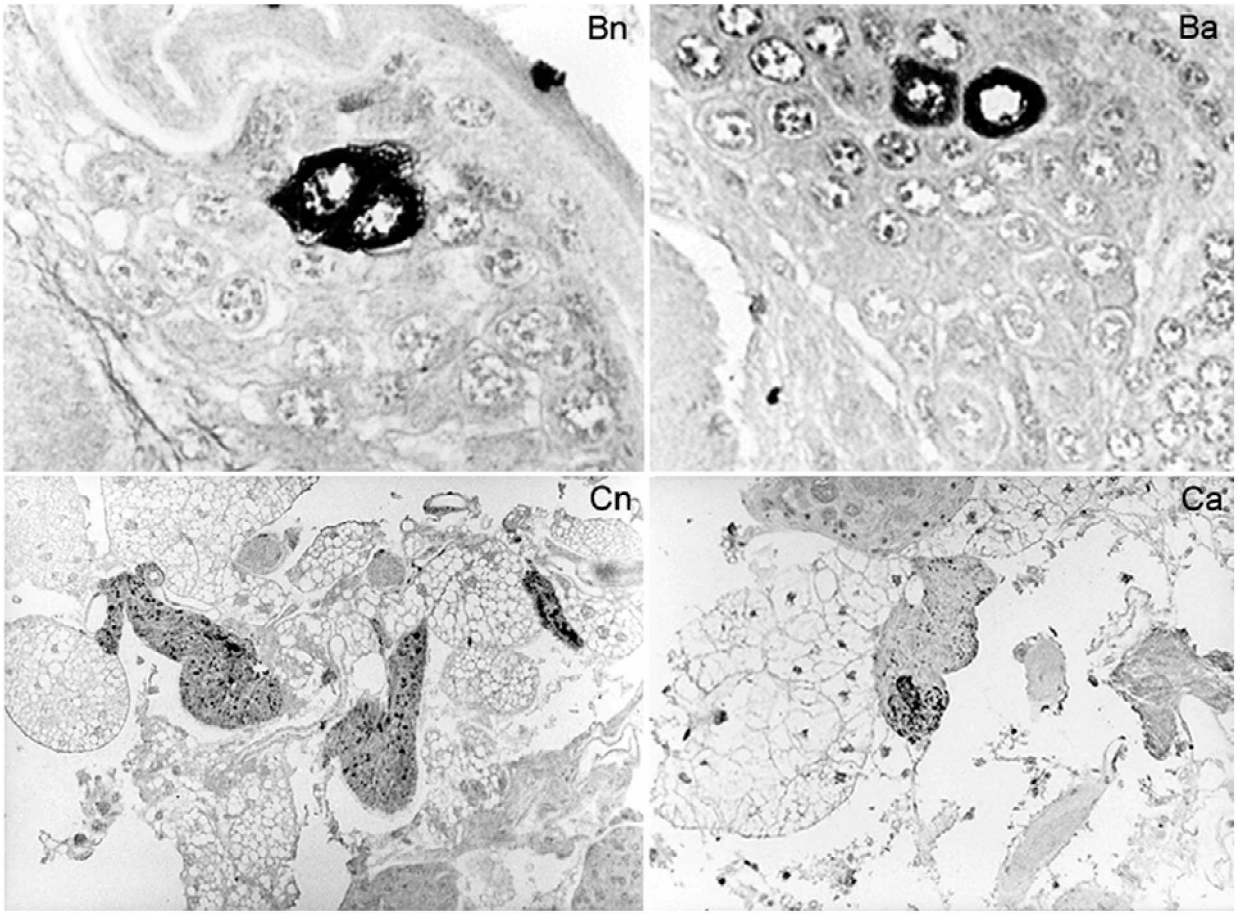

Fig. 1. Cross-sections of the brain (B) and the corpora cardiaca (C) of normal colored (n) and albino (a) *Schistocerca gregaria,* showing [His^7]-corazonin immunoreactive cells and fibres.

Complexes of brains and CC of 22–25 day-old adult albino and normal *S. gregaria* were dissected and fixed in Bouin-Hollande's 10% sublimate solution. After 24 h, tissues were rinsed in distilled water, dehydrated in ethanol, transferred in histosol and embedded in Paraplast. To examine co-localization, alternating sections of 4 µm thickness were made with an LKB Historange microtome (LKB, Uppsala, Sweden) using glass knives.

For immunostaining, the peroxidase-anti-peroxidase (PAP) method as described by Schoofs et al. (1987) was used. Preabsorption of the antisera (diluted 1:2000) with 10 nmol [His^7]-corazonin per ml overnight at 4°C was performed to test the specificity of the antisera.

HPLC analysis of corpora cardiaca (CC)

Thirty pairs of CC were collected from 8–9 day-old albino adults of *S. gregaria* and homogenized and extracted twice in a methanol/water/acetic acid (90:9:1, v/v/v) solution. After centrifugation (10000 g for 5 min), the supernatants were pooled and combined with 400 µl of 0.1% aqueous trifluoroacetic acid (TFA). The methanol was evaporated in a Speedvac concentrator. The aqueous solution was then washed with 300 µl of n-hexane to remove the bulk of lipids. The remaining traces of hexane were evaporated *in vacuo* and the watery layer was filtered through a Millipore PVDF filter (0.45 µm pore size) and used for HPLC analysis. This was performed on a Beckman HPLC system (Programmable Solvent Module 126 connected to a Detector Module 168), with a µ-Bondapak C18 stainless steel column (125Å, 10 µm, 2 × 300 mm, Waters). The gradient used was 0.1% TFA in water for 10 min followed by a linear increase to 50% CH_3CN containing 0.1% TFA in 30 min at a flow rate of 0.5 ml/min. The absorbance was monitored at 214 and 280 nm. Fractions were collected at intervals of 1 min, stored at 4°C and used for the determination of the amino acid sequence. Synthetic [His^7]-corazonin served as a reference.

Determination of the amino acid sequence by mass spectrometry

Nanospray Quadrupole (Q) orthogonal acceleration Time of Flight mass spectrometry was performed on a Q-TOF hybrid ESI-TOF system (Micromass, UK). An aliquot of the HPLC fraction, coeluting with synthetic [His^7]-corazonin, was dried, redissolved in acetonitrile/water/formic acid (50:49.9:0.1, v/v/v) and loaded in gold coated capillaries (Micromass type F nanoflow needles). The sample was sprayed at a flow rate of 30 nl/min, giving extended analysis in which an MS spectrum as well as several MS/MS spectra were obtained. Needle voltage was set at 900 V; the cone voltage was 25 V. During MS/MS or tandem mass spectrometry, fragment ions are generated from a selected precursor ion by collision-induced dissociation (Morris et al., 1996). Argon was used as the collision gas. Since not all peptide ions fragment with the same efficiency, the collision energy is typically varied between 20 and 35 eV so that the parent ion is fragmented into a sufficient number of different daughter ions.

Injection of corpora cardiaca extracts and [His^7]-corazonin

Fifty pairs of CC were dissected from 8–9 day-old normal colored adults of *S. gregaria* and immediately transferred into 500 µl of ice-cold extraction medium (see above). The samples

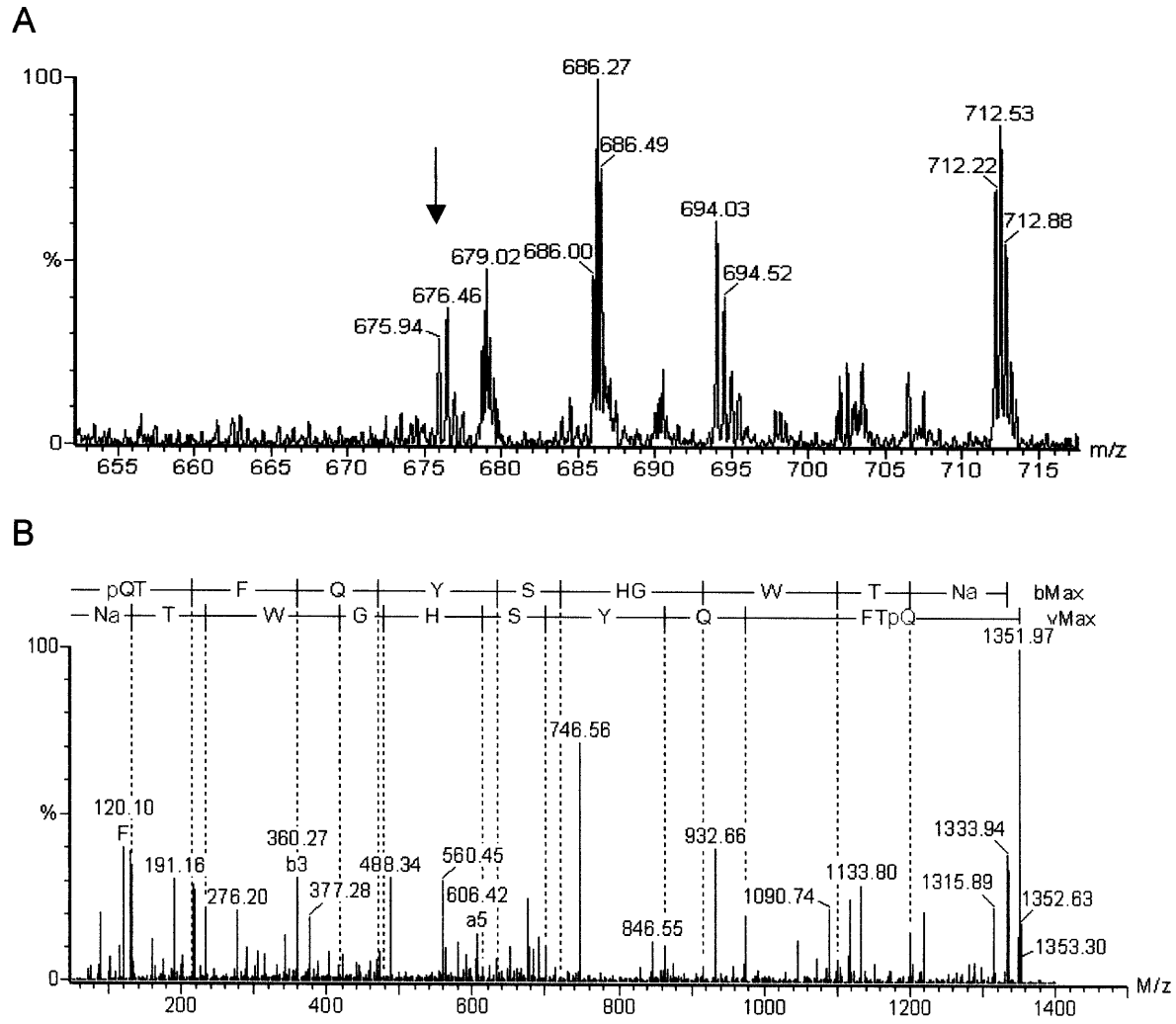

Fig. 2. Q-TOF mass spectrum in MS mode (A) of the HPLC fraction coeluting with synthetic [His^7]-corazonin and collision induced dissociation MS/MS fragmentation spectrum (B) of the doubly charged ion at m/z 675.9 (see arrow).

were sonicated for 15 min and centrifuged at 10000 g for 5 min at 4°C. The supernatant was removed and the pellet re-extracted. Both supernatants were pooled and dried in a Speedvac concentrator and stored in the freezer.

Prior to injection, the dry CC extract was dissolved in 20µl insect saline solution (NaCl 8.766 g/l, $CaCl_2$ 0.188 g/l, KCl 0.746g/l, $MgCl_2$ 0.407 g/l, $NaHCO_3$ 0.336 g/l, sucrose 30.807g/l, trehalose 1.892g/l, pH 7.2). Aliquots of 2 µl, containing 5 CC-equivalents, were injected into mid 4th instar albinos of *S. gregaria* with a Hamilton microsyringe. Synthetic [His^7]-corazonin was dissolved in insect saline and injected into hoppers of the same stage (1 nmol/individual). Controls were injected with insect saline only. As a positive control, albino hoppers of *L. migratoria* were also treated with equal amounts of the peptide. The body color of each individual (10 individuals per treatment) was observed and recorded after their molting to the 5th instar and compared with the controls.

RESULTS

In the brain-CC complex of both albino and normal *S. gregaria*, corazonin immunoreactivity was found in only two pairs of four cells of the pars lateralis in each brain half (Fig. 1). No cells stained in the deutero- and tritocerebrum, in the optic lobes or the suboesophageal ganglion. The CC were also immunopositive. These results reveal that a corazonin-like factor is present in the retrocerebral complex of the albino mutant as well.

The brain-CC extract was analyzed using RP-HPLC. The fraction co-eluting with synthetic [His^7]-corazonin was subjected to Q-TOF mass spectrometry. A doubly charged ion at 675.9 m/z, corresponding to [His^7]-corazonin (1350.9 Da) could be identified (Fig. 2). In a subsequent MS/MS experiment, this ion was selected and fragmented in the collision cell of the Q-TOF. The resulting spectra yielded ions corresponding to the theoretical fragmentation pattern of [His^7]-corazonin. Thus, these findings substantiate the results of the immunocytochemical analysis that suggested that the albino is not deficient in corazonin.

To exclude the possibility that the albinism is due to an insufficient production of endogenous [His^7]-corazonin, 4th instar albino hoppers of *S. gregaria* were injected with 1 nmol of the peptide or with an extract of 5 pairs of CC from normally colored *S. gregaria*. As a positive control, *L. migratoria* albinos were similarly treated with [His^7]-corazonin. None of the *S. gregaria* albinos displayed any darkening of the cuticle, neither in the 4th instar nor after the moult to the 5th instar, whereas corazonin - injected *L. migratoria* albinos turned darker as early as 2 days after the injection and became almost completely black after ecdysis to the 5th instar.

CONCLUSIONS AND DISCUSSION

Mutants represent a powerful tool in unraveling developmental and physiological processes, as clearly shown in *Drosophila* and other model organisms. Likewise, the Okinawa albino strain of *L. migratoria*, which is deficient in [His^7]-corazonin (Tanaka, 1993), is functioning as a promising tool in the further elucidation of the endocrinology of phase transition in locusts (e.g. Hoste et al., 2002a, b). Because *S. gregaria* is a more important pest species than *L. migratoria* and because the majority of research data with respect on phase transition have been obtained with this species, any mutant with an effect on phase transition would be welcome.

For many years, an albino mutant has been inbred by Dr. S.O. Anderson in Copenhagen, Denmark. Schoofs et al. (2000) analysed this mutant for the presence or absence of [His^7]-corazonin. Their conclusion was that albinism in this mutant is not due to a deficiency in [His^7]-corazonin. The albino did not darken upon injection of synthetic corazonin or upon implantation of CC of normally colored *S. gregaria*. However, in the hands of Yerushalmi et al. (2000), some darkening was observed upon injection with a very high dose (50 nmol) of synthetic [His^7]-corazonin into the Denmark mutant. This dose was 10 times higher than ours. They also reported that all enzymes necessary for melanin biosynthesis seemed to be present in the albino strain. The ultimate cause of the absence of melanization was not found.

The albino strain that we established seems to have similar characteristics as the Denmark strain. It can be concluded that it is not deficient in [His^7]-corazonin. Neither does it react to synthetic [His^7]-corazonin by darkening of the cuticle. The defect is apparently situated either at the level of the receptor or in the biosynthetic pathway of melanin. The receptor for [His^7]-corazonin has not yet been identified.

Our immunocytochemical analysis revealed no differences in the staining pattern obtained in the Leuven albino strain as compared to the normal strain and the Denmark albino recently described by Schoofs et al. (2000). [His^7]-corazonin immunoreactivity was found in only a few lateral cells. This corresponds to the situation in *Galleria mellonella*, in which corazonin-positive cells were localized in 2 pairs of neurons in the lateral region of each brain hemisphere (Hansen et al., 2001). In addition, the coding gene has been found in *G. mellonella*. These findings show that the sequence of corazonin in *G. mellonella* (pGlu-Thr-Phe-Gln-Tyr-Ser-Arg-Gly-Trp-Thr-Asn) is identical to all known [Arg^7]-corazonins. The corazonin found in locusts differs only in position 7 and has there a His instead of an Arg. This high degree of conservation suggests that corazonin may play a crucial role in insect endocrinology.

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