Stimulatory Effect of Octopamine on Juvenile Hormone Biosynthesis in Honey Bees (*Apis mellifera*): Physiological and Immunocytochemical Evidence

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The effect of octopamine on the activity of corpora allata of adult worker honey bees has been examined *in vitro* and correlated to the local distribution of this biogenic amine in brain and retrocerebral complex as studied immunocytochemically by means of an highly specific antiserum. Octopamine causes a dose-dependent increase in juvenile hormone release from corpora allata. Maximum increase is obtained with concentrations of 10^{-6} M in nurse and foraging bees by 45.3 or 32.3%, respectively. Octopamine-like immunoreactivity occurs in about 45 somata of the median neurosecretory cells in the pars intercerebralis of the bee brain. They project via immunopositive nervus corporis cardiaci I into the corpora cardiaca, where interspersed varicose structures and 8–10 cell bodies in the ventral part of this gland are stained. A network of immunoreactive fine varicose nerve fibres surrounds each gland cell of the corpora allata. Immunoreactivity in these neuronal structures is detectable if bees were starved over night, a condition in which corpora allata elicit the highest juvenile hormone production ever observed in bees. Both, the stimulatory effect of octopamine and the presence of immunoreactive nerve fibers in the corpora allata, strongly indicate a physiological role of this biogenic amine in the regulation of juvenile hormone biosynthesis in adult honey bees.

Apis mellifera Octopamine Immunocytochemistry Juvenile hormone biosynthesis Corpora allata
Corpora cardiaca Neurosecretory cells

INTRODUCTION

Metamorphosis and reproduction are controlled in most insect species by the juvenile hormones (JH), sesquiterpenoids produced by the corpora allata (CA). Although the innervation of these endocrine glands is anatomically complex and has not been completely described for any species (Thompson and Tobe, 1990), it is known that the nervous system exerts considerable influence on the biosynthesis of JH. The activity of the CA is regulated by a variety of inhibitory and stimulatory humoral and neuronal factors originating mainly from the insect brain (Tobe and Stay, 1985). During the last few years, neuropeptides acting as allatotropins or allatostatins have been isolated from brains of a few insect species (Katoaka *et al.*, 1989; Pratt *et al.*, 1989, 1991; Wood-

head *et al.*, 1989; Kramer *et al.*, 1991; Duve *et al.*, 1993). Besides these allatoregulatory peptides, another group of physiologically active brain substances, the biogenic amines, has been shown to be involved in the regulation of JH biosynthesis. Especially octopamine, that appears to function as a neurohormone, a neurotransmitter and a neuromodulator in insects (Evans, 1985; Agricola *et al.*, 1988; Erber *et al.*, 1993), enhances JH release from CA in the locust *Locusta migratoria* (Lafon-Cazal and Baehr, 1988) and inhibits JH release in the cockroach *Diploptera punctata* (Thompson *et al.*, 1990).

In honey bees (*Apis mellifera* L.) as well as in other hymenoptera, regulatory factors of JH biosynthesis are not yet known, although this hormone plays a central role in the control of queen and worker caste determination (Rachinsky and Hartfelder, 1990) and in the control of the workers age-dependent task performance (Robinson, 1992) and reproduction (Kaatz *et al.*, 1992a; Robinson *et al.*, 1992). The biogenic amines dopamine, noradrenaline, serotonin and octopamine are found in large amounts in the central nervous system of adult honey bees (Mercer *et al.*, 1983; Mercer 1987; Fuchs

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et al., 1989; Brandes *et al.*, 1990; Taylor *et al.*, 1992). Their levels are affected by environmental and genetic factors (Harris and Woodring, 1992) and influence neuronal activity and behavior in adult workers (Bicker and Menzel, 1989; Erber *et al.*, 1993). In the present paper we examined the effect of octopamine on honey bee CA activity and localized the topographical distribution of this major biogenic amine in the brain and the retrocerebral complex using a specific antiserum (Eckert *et al.*, 1992) to gather insight into the complex mechanisms that influence JH biosynthesis, which in turn primarily generates JH titres (Huang *et al.*, 1991) and finally exerts its numerous physiological and morphogenetic functions in honey bees.

MATERIALS AND METHODS

Honey bees

Worker honey bees (*Apis mellifera* L.) were taken from queenright two-storey hives. Adult workers of known age were obtained from combs with sealed brood kept in an incubator at 34.5°C and 80% relative humidity. Newly emerged workers were labelled with coloured markers and introduced into test colonies. Nurse bees were removed from combs with open brood cells and determined by age (6 days) and fully developed hypopharyngeal glands (Maurizio, 1954). Foragers were caught at the hive entrance after returning from foraging flight and recognized by their pollen loads or by paint marks which they received at an artificial food source.

JH biosynthesis

Rates of JH release by the CA were measured by an *in vitro* radiochemical assay originally described by Pratt and Tobe (1974) and modified for application to adult honey bees (cf. Hildebrandt and Kaatz, 1992). Dissection and incubation of CA were carried out in a defined, honey bee-specific culture medium (Kaatz *et al.*, 1985) lacking L-methionine and bovine serum albumin and supplemented with 0.7 mM L-leucine. One or two pairs of glands each were collected in capped glass culture dishes in 50 µl methionine-free medium. After a preincubation period of up to 30 min, the medium was replaced by 50 µl medium containing 100 µM L-[³H-methyl]-methionine (New England Nuclear, sp. act. 7.4 GBq/mmol). Glands were incubated at 34.5°C in the dark and gently shaken (45 rpm) in medium for 3 h to determine basal rates of JH release. Subsequently for another 3 h, the CA were transferred into medium containing brain extracts or octopamine or into control medium. *In vitro* synthesized JH III, which is the only naturally occurring homologue in honey bees (Rembold, 1987), was extracted from the incubation medium via rapid partition assay (Feyereisen and Tobe, 1981) and quantified by liquid scintillation spectrometry.

Brain extracts

Brains of foraging bees were dissected out of the frontally opened head capsule and placed into cold distilled water. After sonication 2 × 30 s on ice, homogenates were centrifuged with 10,000 g at 4°C for 10 min. The pellet was reextracted by sonication and both supernatants were pooled and concentrated under vacuum. The crude extract was stored until use at -70°C.

Immunocytochemistry

Immunocytochemical staining was performed on paraffin sections of the brains and adhering retrocerebral complexes of 160 foraging bees. After capture, workers were immobilized by cooling at 4°C. Brains and retrocerebral complexes were dissected either out of the prefixed head after immediate perfusion with fixative or out of the head capsule and subsequently immersed in the fixative (6% glutaraldehyde and 1% acetic acid in aqueous saturated picric acid). In three separate experimental series with five bees each, foragers were caught in the early afternoon, kept individually at 12°C without feeding over night and dissected in the following morning. The explanted tissues were fixed at room temperature for 4–6 h. Following dehydration and embedding in paraplast, serial sections (12 µm) were cut and mounted on poly-lysine coated slides.

After rehydration, the sections were carefully washed in Tris-buffered saline (TBS: 0.3 M NaCl, 0.1 M Tris-HCl, pH 7.6) and then incubated in sodium borohydride solution [1% NaBH₄ (w/v) in 0.1 M Tris-HCl] at room temperature for 30 min. After three rinses in TBS, sections were incubated either in 5% normal goat serum (NGS) or in the blocking solution of a Vectastain ABC-kit (Vector Laboratories) diluted in TBS plus 0.1% Triton X-100 (TBST) for 1 h. The polyclonal rabbit antiserum against octopamine (cf. Eckert *et al.*, 1992), a generous gift of J. Rapus and Dr M. Eckert (University of Jena, Germany), was diluted 1:800 in TBST plus 1% NGS and applied on the sections at room temperature in a humidity chamber over night. Unbound serum was removed by three rinses with TBST. Specific binding of the antiserum was detected either by the PAP technique (Sternberger, 1979) by incubation in goat anti-rabbit immunoglobulin G (Sigma, 1:40 in TBST, 1 h) followed by three washes in TBST and by incubation with rabbit peroxidase-antiperoxidase complex (Dakopatts, 1:100 in TBST, 1 h). Alternatively, sections were treated according to the protocol given with the Vectastain ABC-Kit using TBST for the biotinylated secondary antibody and TBS for the ABC complex as dilution buffers. After washing in 0.1 M Tris-HCl (pH 7.6, 3 × 10 min), sections were treated with 3,3'-diaminobenzidine solution (Sigma, 0.2 mg/ml in Tris-HCl with 0.001% H₂O₂, 5–15 min). Finally, sections were washed, dehydrated in an ethanol series, cleared in xylene and mounted in Entellan (Merck).

Sections were viewed and photographed with a Poly-

var microscope (Reichert-Jung). Schematic presentations were drawn from serial sections with the aid of a camera lucida.

Octopamine-like immunoreactivity in the CA was mapped with a computer aided microscope digitizer system (AutoCAD). The three dimensional pattern of stained structures was reconstructed from drawings of the successive serial sections. CA surfaces and vertically extending nerve processes were used to align adjacent sections. The 3-D reconstruction was then printed by generating two views with 3° declination on the 3-D object.

Specificity controls

The specificity of the employed anti-octopamine serum, raised in rabbits using octopamine coupled to thyroglobulin with glutaraldehyde, was originally characterized in the cockroach *Periplaneta americana* by Eckert *et al.* (1992) and meanwhile tested in several other arthropods (Stevenson *et al.*, 1992; Nürnberger *et al.*, 1993; Schneider *et al.*, 1993). It does not crossreact with dopamine, noradrenaline and serotonin. For honey bee brain and retrocerebral complex sections, we performed the following controls: (a) incubation in the absence of primary antiserum; and (b) injection of 1 µg reserpine per control bee to remove all biogenic amines from nervous tissue (Braun and Bicker, 1992) 12 h prior to dissection and complete immunocytochemical staining. Immunoreactivity was abolished by all control treatments.

RESULTS

Effects of octopamine on JH production

The kinetics of *in vitro* incorporation of the ³H-methyl moiety of methionine into JH by the CA was studied in nurse and foraging bees. After a lag phase of 20 min, both stages released JH at constant rates for 6 h with 1.64 (nurse bees) and 5.30 pmol JH·pair CA⁻¹·h⁻¹ (foragers) (Fig. 1). Therefore we chose two successive incubation periods of 3 h each for the following experiments.

Treatments with 10⁻⁶ M octopamine significantly stimulated production of JH by CA from nurse bees and foraging bees by 45.3 and 32.3%, respectively, relative to the untreated controls (Fig. 2), but the enhanced JH production in young queenless workers was not affected (data not shown). Crude brain extracts from foraging bees resulted in an even higher increase of CA activity in the latter stage (60.4%). The 15–20% increase of JH release in both control groups during the second incubation period is due to the lag-phase occurring *in vitro* during the first incubation period (cf. Fig. 1). Octopamine affected CA activity in a dose-dependent manner (Fig. 3). The threshold for this response laid between 10⁻⁷ and 10⁻⁸ M. Maximum stimulation in foraging bees occurred at 10⁻⁶ M octopamine, but the effects by 10⁻⁵ and 10⁻⁷ M octopamine, though weaker, did not differ significantly (U-test, *P* < 0.05).

FIGURE 1. Kinetics of JH release by CA of nurse (▲) and foraging (●) bees *in vitro*. Each point represents the mean $\pm$ SEM of 12–24 single pairs of CA. For the short time incubations (10–30 min), up to 6 pairs of CA were coincubated in order to detect even minute amounts of released JH.

Octopamine-like immunoreactivity

In order to analyse the occurrence and local distribution of octopamine in those parts of the nervous system that are considered to be involved in the regulation of JH biosynthesis in insects, we employed a specific antiserum against octopamine on serial sections of bee brains and retrocerebral complexes. Octopamine-like immunoreactivity was reproducibly detectable in the median neurosecretory cells (MNC), nervus corporis cardiaci (NCC) I, somata and varicose fibres of the corpora cardiaca (CC) and nerve fibres surrounding the glandular cells of the CA of foraging bees, which were kept without food at 12°C over night [Fig. 4(a)]. In bees directly removed from the hive, immunolabelling of the above mentioned neuronal structures occurred in less than 5% of all analysed bees.

FIGURE 2. Stimulatory effect of octopamine and crude brain extracts on JH III biosynthesis of nurse and foraging bees. Two pairs of CA were coincubated for 3 h in normal medium to establish untreated rates of JH biosynthesis (open bars) and then incubated for 3 h with 10⁻⁶ M octopamine or crude brain extracts (hatched bars). The results were compared with a control group receiving only normal medium for both periods. Significant differences are denoted by different letters above the data columns (U-test, *P* < 0.05). Mean $\pm$ SEM, *n* = 8.

FIGURE 3. Dose-dependent effect of octopamine on JH release from CA of foraging bees. Same experimental conditions as described in Fig. 1. The percent change, relative to untreated controls during first incubation, is shown. Each point represents 6–12 determinations (mean + SEM, two pairs of CA per incubation).

Approximately 45 somata of the 120 median neurosecretory cells in the pars intercerebralis were immunoreactive [Fig. 4(b)]. These cells are ventrally located in the MNC cluster and are sporadically interspersed by unlabelled perikarya. Dorsally located MNC just as lateral neurosecretory cells and their respective nerve projections, the NCC II, were never labelled with the antibody. Immunoreactivity was also visible in the protocerebral central body [Fig. 4(b)].

Processes leaving the cell bodies of the labelled MNC project via immunolabelled NCC I into the CC [Fig. 4(b)]. The CC contain densely packed immunoreactive varicose structures in the ventral half [Fig. 4(c and d)]. In their immediate vicinity we found 8–10 intensely stained ellipsoid somata with unstained nuclei. They are smaller ($<5\ \mu\text{m}$ wide) than and separated from the unlabelled relatively large intrinsic glandular corpus cardiacum cells ($>10\ \mu\text{m}$ across) and their cell processes were hardly discernible [Fig. 4(d)]. Immunolabelled varicose fibres also terminated in the most dorsally located group of large glandular cells.

In the CA very fine octopamine-immunoreactive nerve fibres with varicose terminals surround each of the gland cells forming a branched neuronal network in the gland tissue [Fig. 4(e and f)].

Starvation and JH biosynthesis

Since octopamine-like immunoreactivity was consistently obtained when bees were starved, we compared CA activity of bees kept under such experimental conditions with those in normal colonies. JH release increased 1.81 times in foragers and even 7.07 times in nurse bees (Fig. 5), reaching maximum mean rates of 9.05 and 10.11 pmol JH·pair CA⁻¹·h⁻¹ *in vitro*, respectively, far above the values, which were ever observed in bees under normal colony conditions.

DISCUSSION

Our results clearly demonstrate that octopamine stimulates JH release by explanted CA of adult honey bees. Since JH release is linearly correlated with JH biosynthesis by CA *in vitro* and reflects the JH titres in the haemolymph (Huyang *et al.*, 1991), it provides an accurate estimate for JH biosynthesis in adult worker honey bees. Thus, we assume that this biogenic amine also stimulates biosynthesis of JH *in vivo*. Octopamine effects on JH release have been previously only demonstrated in a locust (Lafon-Cazal and Baehr, 1988) and in a cockroach (Thompson *et al.*, 1990). The bell shaped dose-response curve shows striking similarities to that of octopamine on the neuronal activity in the visual system of the honey bee (Erber *et al.*, 1991). Octopamine concentrations above 10^{-4} M are ineffective in both target tissues. The reason for the observed ineffectiveness, however, remains a matter for speculation. Since serotonin is the prominent antagonist of octopamine in the bee (Erber *et al.*, 1993), one might argue, among numerous other possibilities, that octopamine acts in high concentrations on serotonergic receptors that mediate inhibition of JH biosynthesis.

The observed effect of octopamine on CA activity coincides with the octopamine-like immunoreactivity which was found for the first time in insects in some of the neuroendocrine tissues such as neurosecretory cells and CC that are considered to be involved in the regulation of JH biosynthesis (Tobe and Stay, 1985; Thompson and Tobe, 1990; Duve *et al.*, 1991; Stay *et al.*, 1992). To date octopamine was found to be present in insect neurosecretory tissues only by the use of biochemical methods (cf. David and Coulon, 1985). Octopamine-like immunoreactivity, however, was recently detected in segmentally arranged neurosecretory cells of the ventral nerve cord in lobsters (Schneider *et al.*, 1993). By means of the same antibody we found an immunoreactive group of about 45 of the 120 cells of the median neurosecretory cell complex in the Pars intercerebralis of the bees. But the recently discovered lateral neurosecretory cells (Eichmüller *et al.*, 1991) and their nerve projections did not react immunocytochemically. The MNC do not bind considerable amounts of [³H]octopamine as studied with autoradiographic methods (Scheidler, 1991) and, thus, are probably not target sites for this amine. It appears that the immunopositive MNC project via NCC I into the CC. The NCC I fuses in the ventral protocerebrum with the lateral and median fibres of the NCC II to form the NCC of the honey bee (Eichmüller *et al.*, 1991).

We detect heavily stained varicose structures and somata in the ventral part of the CC. The relatively small cells have not been described before and differ in size and shape from the large intrinsic glandular cells especially those 24, which contain adipokinetic hormone-like peptides in *Apis* (Schooneveld *et al.*, 1987). Octopaminergic innervation of the CC has been proposed in insects in the light of biochemical evidence for the presence of octo-

FIGURE 4. Immunocytochemical reactivity of the median neurosecretory cells and the retrocerebral complex to antiserum against octopamine. (a) Summary reconstruction of octopamine-like immunoreactivity in median neurosecretory cells and retrocerebral complex. Posterior view. Fibres originating from the median neurosecretory cells in the pars intercerebralis project into the corpora cardiaca via NCC I. (b) Median sagittal section documents immunolabelling of large sized somata of median neurosecretory cells. Labelled fibres originating in these cells project into the corpora cardiaca via NCC I (arrows). Scale bar: 50 μ m (c) Retrocerebral complex. Scale bar: 50 μ m. (d) Corpus cardiacum lobe with immunolabelled varicose structures and somata (arrow). Scale bar: 10 μ m. (e and f) CA with immunoreactive nerve fibres (arrowheads) surrounding the glandular cells. Scale bar: 10 μ m. (e) Computerized 3-D reconstruction. Abbreviations: al, antennal lobe; ca, corpora allata; cb, central body; cc, corpora cardiaca; mb, mushroom bodies; MNC, median neurosecretory cells; NCC I, nervus corporis cardiaci I; oes, oesophagus; po, pons of the central body complex (protocerebral bridge); rn, recurrent nerve; sog, suboesophageal ganglion; vnc, ventral nerve cord.

FIGURE 5. Effect of starvation on JH production in nurse and foraging bees. Bees were caught and kept individually without feeding at 12°C over night. In the following morning, CA of individual bees were explanted and incubated by pairs. Treatment results in a significant increase of JH release (hatched bars) compared to untreated controls (closed bars) in both stages (U-test, $P < 0.05$, mean $\pm$ SEM, $n = 12$).

pamine in the CC (David and Lafon-Cazal, 1979; Orchard *et al.*, 1986) and the pharmacological evidence for the involvement of octopamine in the release of neuropeptides from the intrinsic glandular cells (Pannabecker and Orchard, 1986). Strongest evidence for an involvement of octopamine in the regulation of CA activity, however, originates from an octopamine-like immunoreactive neuronal network surrounding each glandular cell of the CA. By solely following the immunocytochemical staining in the fine fibres seen in the CA we could not conclusively decide whether some of the octopamine-like neurons in the pars intercerebralis, that extend axons to the CC, innervate the CA, or whether the intrinsic immunopositive neurons in the CC have terminals in the CA, or both. Octopaminergic innervation of the CA in insects has been proposed earlier on the basis of the biochemical detection of octopamine in the CA of locusts (Evans, 1978; David and Lafon-Czal, 1979) and cockroaches (Evans, 1978) and recently based on electrophysiological experiments demonstrating that electrical stimulation of the NCA as well as octopamine treatment elicits hyperpolarization of CA cells, which can be inhibited by the octopamine receptor blocker phentolamine (Thompson *et al.*, 1990).

The present first detection of octopamine-like immunoreactive structures in the neuroendocrine system in insects is without a doubt dependent on the starvation of the bees. Previous approaches in untreated cockroaches (H. Agricola, personal communication) and locusts (Konings *et al.*, 1988) failed. P. A. Stevenson (personal communication), however, detected immunopositive MNC in *Locusta migratoria* once these animals were starved. Starvation is known to increase octopamine levels in the brain of the locust *Schistocerca gregaria* (Davenport and Evans, 1984). Elevated levels of octopamine were also

found in a number of insects including honey bees in response to stress (cf. Harris and Woodring, 1992) and seem to prepare the animal for intense activity (Corbett, 1991). Thus, it is likely that the amount of octopamine in MNC, CC and CA is elevated due to starvation above the detection threshold of the applied immunocytochemical method. The sensitivity of the immunostaining with the antibody as studied in the lobster nervous system amounts to 14 fmol per cell body (Schneider *et al.*, 1993). In the light of our experiments on the explanted CA may such an increase in octopamine content, supposing the amine is released, result in an enhanced JH production. Such an increase in CA activity is actually detectable in starved bees reaching a level that is higher than ever found in any behavioural and physiological state in *Apis* (Hildebrandt and Kaatz, 1990, 1992; Rachinsky and Hartfelder, 1990; Huang *et al.*, 1991; Huang and Robinson, 1992; Kaatz *et al.*, 1992b; Robinson *et al.*, 1992). The observed increase in JH production in both nurse and foraging bees after starvation may accelerate the transition of nurse bees to foraging bees or intensify foraging activity, respectively, as JH is involved in the control of age polyethism in worker bees and treatment with JH, JH mimics or analogues induces precocious foraging. Especially in colonies that lack, due to experimentation, foragers, nurse bees turn within 2 days into active foragers after a premature rise in JH haemolymph titres (cf. Robinson, 1992).

Both, physiological and immunocytochemical, observations suggest that octopamine plays a role in the control of JH biosynthesis by the CA *in vivo*. The present experiments, however, have not determined whether octopamine affects the activity of the CA neuronally via (i) direct effect on the intrinsic cells of the CA, and/or (ii) modulation of the release of neurotransmitters or neurohormones from the many nerve terminals in the CA, or (iii) humorally via circulating levels of octopamine. Evidence from other insects is not yet conclusive either. A direct octopaminergic innervation of the CA was already proposed by Thompson *et al.* (1990), based on the finding that hyperpolarization of the CA, evoked by electrical stimulation of the NCA or by octopamine, was blocked by the α -adrenergic receptor blocker phentolamine in *Diploptera punctata*. The present immunocytochemical evidence substantiates this hypothesis as it suggests that octopamine is involved in the neuronal control of the CA activity in *Apis* probably being released in the immediate vicinity of the adjacent gland cells. But octopamine is presumably not the sole allatotropin acting on JH biosynthesis in bees, since it stimulates JH release of explanted CA from foraging bees merely by 32%, whereas brain extracts enhance JH production by 60%. This indicates that other, yet unknown factors besides this amine are involved in the stimulation of CA activity. Also, the large increase in the rates of JH biosynthesis after starvation (Fig. 5), especially in the case of nurse bees, is probably not only due to an octopamine exposure *in vivo*. Primary candidates are allatotrophic neuropeptides which have been recently characterized in *Manduca sexta* (Kataoka

et al., 1989). They may directly affect JH biosynthesis or interact with octopamine. Their release from putative peptidergic neurons could be modulated by octopamine, since it is known that this amine is able to mediate neuropeptide release in other insects (Pannabecker and Orchard, 1986). Although octopamine-like immunoreactivity found in nerve terminals of the CA suggests a neuronal control, JH biosynthesis could also be controlled, at least in part, by circulating levels of octopamine. Especially since octopamine is released into the haemolymph in insects, acts as a neurohormone and is elevated especially during stress and flight (cf. Orchard *et al.*, 1993). Such a humoral mode of action would coincide with the detected octopamine-like varicosities and somata in the CC that presumably function as neurohaemal organs. Future studies will elucidate the functional significance of octopamine as a neuromodulator controlling the release of allateregulatory neuropeptides or as a neurohormone, directly altering the JH production.

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