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Effect of tyramine and stress on sex-pheromone production in the pre- and post-mating silkworm moth, *Bombyx mori*

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Abstract

Tyramine (TA) increased significantly after mating, whereas there were no significant differences in octopamine (OA) and dopamine (DA) levels in the brain–suboesophageal ganglion (SOG) complexes between virgin and mated females. The effects of various biogenic amines were tested on pheromone production of virgin and mated females of the silkworm moth, *Bombyx mori*. After 8 h a significant reduction by TA (46%) was observed. Meanwhile, when OA or DA was injected, a significant increase of pheromone titer was observed in both virgin and mated females. This study also presents evidence for an increase in levels of OA and DA in the brain–SOG complexes in response to mechanical stress in *B. mori* female. TA suppressed pheromone production in an *in vitro* pheromone gland (PG) homogenate preparation, thus suggesting that the target of TA is the PG. TA inhibited pheromone production *in vitro* in a dose-dependent manner and DA had a lower inhibitory activity than TA, whereas OA had no effect, suggesting that TA is a candidate for regulating pheromone production in the PG, although other factors could be responsible for the pheromonostatic function.

Keywords: Stress; Sex pheromone; PBAN; Tyramine; *Bombyx mori*; Bombykol

1. Introduction

Biogenic amines such as dopamine (DA), octopamine (OA) and tyramine (TA) are widely distributed in the central nervous system of insects (Evans, 1980). The administration of biogenic amines and agonists or antagonists for their receptors and direct measurement of concentrations of biogenic amines under various conditions indicate that these agents function as neurotransmitters, neuromodulators and neurohormones. They are profoundly involved in regulating many physiological phenomena such as learning (Dudai, 1986), memory (Yovell and Dudai, 1987), circadian rhythms (Muszynska-Pytel and Cymborowski, 1978), contraction rhythm of muscles, flight (Goosey and Candy, 1980), walking, feeding behavior (Long et al., 1986) and mating behavior (Giebultowic et al., 1990), and in the insect's reaction to various stress stimuli (Davenport and Evans, 1984).

Female moths emit a species-specific male attractant (sex-pheromone blend) from a glandular area at the abdominal tip. Sex-pheromone production in many species of moths is controlled by pheromone biosynthesis activating neuropeptide (PBAN) or PBAN-like factors (Raina, 1993). To date, PBANs have been isolated and their primary structures were determined from following species of moths: *Helicoverpa zea* (Raina et al., 1989), *Bombyx mori* (Kitamura et al., 1989, 1990), *Pseudaletia separata* (Matsumoto et al., 1992), *Lymantria dispar* (Masler et al., 1994) and *Agrotis ipsilon* (Duportets et al., 1998). PBAN, which acts on pheromone glands to stimulate pheromone biosynthesis (Rafaeli, 2002), was first identified as a 33-amino acid C-terminal amidated peptide (Raina et al., 1989). Subsequently, it was determined that the five C-terminal amino acids, FXPRLamide, represented the minimal sequence required for activity (Raina and Kempe, 1990). This motif has been identified

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from a variety of peptides and most will stimulate pheromone biosynthesis when tested on pheromone glands isolated from moths (Ma et al., 1996).

A temporary or permanent suppression of pheromone production after mating has been reported for many species of moths. Reports on this process show that different mechanisms are used by different species of Lepidoptera. In a few species of *Helicoverpa* moths, factors originating from the testis or the accessory gland of the male reproductive system and acting through the mated female's haemolymph, are implicated in suppressing pheromone production (Raina, 1989; Ramaswamy et al., 1994). A polypeptide with such pheromonostatic activity has been identified in the male-accessory glands of *H. zea* (Kingan et al., 1993, 1995). It is not clear what role is played by the pheromonostatic peptide. In the gypsy moth and two tortricid moths, suppression of pheromone production after mating seems to be induced by a neural signal that originates from the abdomen and runs through ventral nerve cord (VNC) to inhibit the release of PBAN, because transection of the VNC fails to induce the post-mating inactivation of pheromone production (Giebultowic et al., 1990; Foster, 1993; Jurenka et al., 1993; Foster and Roelofs, 1994). In the silkworm moth, *B. mori*, a decrease in pheromone titers after mating seems to be caused by the same mechanism (Ichikawa et al., 1996a). Mating inhibits the release of PBAN into the haemolymph and a *Drosophila*-like sex-peptide is involved (Wedell, 2005). The neural signal travelling through the VNC to the brain–suboesophageal ganglion (SOG) complexes triggers an inactivaion of PBAN-neurosecretory cells present in the SOG (Ichikawa, 1995; Ando et al., 1996; Ichikawa et al., 1996b). However, the neural mechanism remains to be clarified.

Post-mating inactivation of pheromone production is inhibited or recovered by application of stresses, such as restrictions and anesthesia, in *B. mori* (Ichikawa et al., 1996a). The elucidation of such phenomenon could lead to clarification of the neural mechanism. In this study, a relationship between sex-pheromone production and biogenic amines in brain–SOG complexes in *B. mori* was investigated using reversed-phase high-performance liquid chromatography (HPLC) with electrochemical detection (ECD). The changes in OA, DA and TA levels in brain–SOG complexes relative to mating stimuli and mechanical stress were observed. Futhermore, the effects of external DA, OA and TA on sex pheromone (bombykol) production of *B. mori in vivo* and *in vitro* at its candidate target organ, the pheromone gland (PG), were examined.

2. Materials and methods

2.1. Chemicals

Synthetic PBAN-I from *B. mori*, obtained from Peninsula Laboratories, Inc. (Belmont, CA) or Phoenix Pharmaceuticals, Inc. (Belmont, CA), was dissolved in distilled or deionized water in suitable concentrations. DA hydrochloride, DL-OA hydrochloride, sodium 1-octanesulfate (SOS), perchloric acid (PCA), TA, methanol, hexane and 2-propanol for HPLC, yohimbine hydrochloride and ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate were obtained from Nacalai Tesque, Inc. (Kyoto, Japan); serotonin (5-HT) creatinine sulfate from E. Merck AG (Darmstat, Germany); Tris–HCl, MgCl, sucrose, pepstatin A, aprotinin, leupeptin, benzamidine, PMSF, NADPH, ATP, CoA and 3,4-dihydroxybenzylamine (DHBA) hydrobromide from Sigma Chemical Co. (St. Louis, MO); DL-epinephrine (E) hydrochloride from Tokyo Chem. Ind. Co. Ltd. (Tokyo, Japan); and DL-norepinephrine (NE) hydrochloride from Janssen Chemica (Beerse, Belgium).

2.2. Insects

Pupae of *B. mori* (hybrids of Kinshu × Showa) were kept at 25 °C under a 16:8 light–dark photoperiod. Adults emerging from the pupae were collected each day and kept under the same conditions. Approximately 7 h after the beginning of the second phtophase, when the pheromone titer is maximal (Ando, 1988), a female was mated with a 1 or 2-day-old male. The couple was forced to separate 3 h after the start of mating. Although mating usually continues for more than 24 h and 3 h of mating is required to completely stop pheromone production.

2.3. Surgical operations and application of stressors

The VNC of virgin females was transected on day 0 (the day of eclosion). A drop of melted wax was placed on the ventral side of the fourth abdominal segment and peeled to remove scales. The females were anesthetized in crushed ice for 15 min. A small incision was made on the operation area, and the VNC was cut using micro-scissors. For sham-operated controls, females were treated similarly except for the severance of the VNC. Penis-less males were obtained by surgical operation. After the operation, the wounds were treated with melted wax. Mechanical stress was applied to females by clipping the wing on an acrylic plastic board (0.5 mm thick).

2.4. Analysis of bombykol titers

TA, DL-OA hydrochloride, DA, E, NE and 5HT were dissolved in distilled or deionized water at suitable concentration (1 mM) and at 4 h after the onset of the second photophase, 10 μl of the test solutions or H_2O (control) were injected into abdomen of females using a 10 μl micro-syringe. The PGs were excised and bombykol was extracted by soaking each gland in 500 μl of hexane for 10 min at ambient temperature. The extracts were directly injected onto an HPLC column (150 × 4.6 mm i.d., 5-μm average particle size, Nucleosil 5NO2, Chemco Pak, Osaka, Japan or 250 × 4.6 mm i.d., 5-μm average particle