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Peripheral regulation by ecdysteroids of olfactory responsiveness in male Egyptian cotton leaf worms, *Spodoptera littoralis*

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ABSTRACT

Physiological and behavioral plasticity allows animals to adapt to changes in external (environmental) and internal (physiological) factors. In insects, the physiological state modulates adult behavior in response to different odorant stimuli. Hormones have the potential to play a major role in the plasticity of the olfactory responses. To explore if peripheral olfactory processing could be regulated by steroid hormones, we characterized the molecular, electrophysiological, and behavioral response to changes in endogenous hormone levels in adult male *Spodoptera littoralis*. The expression of the receptor complex (EcR/USP) was localized by in situ hybridization in the olfactory sensilla of antennae. Injections of 20-hydroxyecdysone (20E) induced an ecdysteroid signaling pathway in antennae and increased expression of the nuclear receptors EcR, USP and E75. Diacylglycerol kinase (DGK) and CaM expression were also up-regulated by 20E. Taken together, these molecular, electrophysiological, and behavioral results suggest a hormonal regulation of the peripheral olfactory processing in *S. littoralis*.

1. Introduction

Physiological and behavioral plasticity are critical for the adaptation to fluctuating environmental (e.g. light or temperature) and life history (e.g. mating status or age) factors. The physiological state is one important intrinsic factor modulating adult behavior in response to different odorant stimuli. However, the role that the hormones might play in the plasticity of the olfactory system is not totally understood. In vertebrates, olfactory sensitivity to and behavioral preference for odorants are modulated by steroids (for review, Moffatt, 2003). In female rats and mice, cyclic changes in ability to detect weak olfactory stimuli are correlated with the estrous cycle and ovarian hormone levels (Pietras and Moulton, 1974). Similarly, in male rodents, androgens modulate olfactory preferences for female chemosignals (Stern, 1970; Bean et al., 1986). In insects, physiological state affects how adults respond to odorant stimuli, such as pheromones, food, or host plant odors (Browne, 1993). In adult locusts (Schistocerca gregaria) and male moths (Agrotis ipsilon), juvenile hormone (JH) plays a role in the modulation of olfactory processing (Ignell et al., 2001; Anton and Gadenne, 1999).

In arthropod development, ecdysteroids control developmental processes, such as molting and metamorphosis. The ecdysteroid signaling pathways include nuclear receptors acting as transcriptional factors. The ecdysteroid nuclear receptor complex is a heterodimer of the ecdysone receptor (EcR) and ultraspiracle (USP) (Koelle et al., 1991; Yao et al., 1992). This receptor complex induces the expression of early genes, such as E75 (Asburner, 1974; King-Jones and Thummel, 2005). In adults, ecdysteroids have roles in reproduction, such as spermatogenesis and the development of the male reproductive tract and the associated accessory glands (for review, Brown et al., 2009). However, little information is known about how these hormones affect olfactory-mediated behavior. What is known is restricted to the development (Vogt et al., 1993; Kraft et al., 1998; Schachtner et al., 2004; Franco et al., 2007) and remodeling (Lee et al., 2000; Kuo et al., 2005; Marin et al., 2005) of olfactory central neurons in larvae, pupae, and rarely adults (Cayre et al., 2000). Although some studies suggest that physiological state plays a role in peripheral modulation of olfactory-mediated behavior (Martel et al., 2009; Halem et al., 2001; Wang et al., 2003), the role of hormones on peripheral olfactory processing remains unknown.

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In adult male *Spodoptera littoralis*, ecdysteroid levels in the haemolymph and testes are controlled by the circadian clock (Polanska et al., 2009). The decline of ecdysteroid levels from the photophase to the scotophase corresponds to sperm release, when *S. littoralis* has high mating activity, female pheromone production/release, and male pheromone responsiveness (Dreisig, 1986; Silvegren et al., 2005). Likewise, an antennal circadian clock rhythm in pheromone reception has been observed in *S. littoralis* (Merlin et al., 2007).

Odorant molecules are detected by olfactory receptor neurons (ORNs) located in antennal sensillae. The molecular mechanisms of the odorant transduction remain controversial, since it is not yet clear whether olfactory receptors are coupled or not to G proteins (Nakagawa and Vosshall, 2009). Phospholipid signaling is thought to be involved in the olfactory transduction (Lucas and Pézier, 2006; Kain et al., 2008; Stengl, 2010). In *S. littoralis*, diacylglycerol kinase (DGK), which phosphorylates DAG to produce phosphatidic acid (Sakane and Kanoh, 1997), is expressed antennae, likely in ORNs (Chouquet et al., 2008). DGK might participate in or modulate olfactory transduction in insects (Kain et al., 2008), as it modulates in rodent vomeronasal sensory neurons (Lucas et al., 2003).

In vertebrates and insects, Calmodulin (CaM), a ubiquitous Ca^{2+} sensor protein, also plays an important role in olfactory processing by activating the kinase cascade or by regulating ion channels, such as Ca^{2+} channels, IP_3 receptor, and cyclic nucleotide gated (CNG) channels (Saimi and Kung, 1994; Zheng et al., 2003; Pifferi et al., 2006; Matsumoto et al., 2006; Krannich and Stengl, 2008; Spehr et al., 2009). In vertebrates, steroids increased CaM's propensity to release Ca^{2+} (Zylinska et al., 2009).

To demonstrate that the peripheral olfactory processing can be regulated by ecdysteroids in insects, we studied the ecdysteroid signaling pathway, the CaM and DGK expression in antennae, and the electrophysiological response during pheromonal stimulation. Our results showed that steroid hormones modulated the peripheral olfactory processing which is implicated in the reproduction of *S. littoralis*. This modulation could involve the regulatory proteins, CaM and DGK.

2. Materials and methods

2.1. Animals, tissue collection and hormone treatments

S. littoralis were reared on a semi-artificial diet (Hinks and Byers, 1976) at 24 °C, 60–70% relative humidity and under a 16:8 light: dark cycle until emergence. They were sexed as pupae. In the middle of the scotophase, adult tissues (antennae, brain, eyes, thorax, thoracic muscles, legs, abdomens, gut) were dissected, frozen in liquid nitrogen, and stored at −80 °C until RNA isolation. For in situ hybridization, male antennae were cut into 5–6 pieces and fixed overnight in 4% paraformaldehyde (PFA) at 4 °C, dehydrated in methanol, and stored at −20 °C until use. 20E (gift from Pr. René Lafont, UPMC, Paris, France) was prepared as a stock solution (5 μg/μL) and diluted to 1, 10 and 100 ng/μL in an NaCl (145 mM) solution. Before the onset of the scotophase, 2-day-old males (0.11 ± 0.02 g body weight, $N = 30$) were injected in the abdomen with a Hamilton syringe (700 RN serie, capacity: 25 μl, gauge: 22 S) with 2.5 μL of one of the three dilutions. Control insects were injected with 2.5 μL of the NaCl (145 mM) solution. For the dissections, the electrophysiological and the behavioral experiments, insects were used 12 h after 20E injection.

2.2. Ecdysteroids extraction and determination in haemolymph

To collect haemolymph, the top of an insect's head was cut, and the whole animal was placed in a 0.2 mL tube with a hole in the bottom. This tube was then placed inside a 1.5 mL tube and the assembly was centrifuged at 500 g for 10 min. Haemolymph pooled from five insects from the same 20E treatment was suspended in ten volumes of methanol (purity 99.9%, MERCK, France) and centrifuged at 10,000 g for 10 min. The supernatant was collected and dried under vacuum in a SpeedVac Concentrator (Eppendorf, France). Samples were resuspended in buffer for an enzyme immunoassay (EIA) for ecdysteroid, adapted from Porcheron et al. (1989), using polyclonal anti-20E antiserum AS4919 and a 2-Succinyl-20-hydroxyecdysone coupled to peroxidase (Gift from Jean-Paul Delbecque, Bordeaux, and Catherine Blais, Paris). The chromogenic reaction used o-Phenylenediamine (OPD, Sigma, France) and hydrogen peroxide solution (Mesnier et al., 2000), and Optical Density (OD) was measured at 450 nm. In our assay, ecdysone and 20E cross-reacted equally well with the AS4919 antiserum. Calibration curves were generated with 20E (range 31–4000 fmol) and the data are expressed as 20E pg/μL of haemolymph. All assays were performed four times.

2.3. RNA isolation and cDNA synthesis

Total RNAs were extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA), treated with DNase I (Ambion, USA) in accordance with the manufacturer's instructions, and quantified by spectrophotometry at 260 nm (BioPhotometer, Eppendorf, Hamburg, Germany). RNA quality was checked by electrophoresis on formaldehyde-agarose gel (1%). Single-stranded cDNAs were synthesized from total RNAs (5 μg) from each tissue with Superscript II reverse transcriptase (Gibco BRL, Invitrogen). The reaction contained a dNTP mix and $Oligo(dT)_{18}$ primer. The mix was heated at 95 °C for 5 min before adding the enzyme, RNAse OUT and sterile water to a final volume of 20 μL. This mixture was incubated for 50 min at 42 °C and 10 min at 70 °C according to the manufacturer's protocol. For 5′ and 3′-RACE PCR, antennal cDNAs were synthesized from 1 μg male antennal RNA at 42 °C for 1.5 h using SMART™ RACE cDNA Amplification kit (Clontech) with Superscript II reverse transcriptase (Gibco BRL, Invitrogen), 5′ or 3′-CDC-primer and SMART II oligonucleotide.

2.4. Cloning of SlEcR, SlUSP, SlE75 and SlCaM

Partial and total cDNAs encoding putative ECR, USP, E75 and CaM were identified from a *S. littoralis* male antennal EST library (Jacquin-Joly et al., 2007) by local TBLASTN analysis in BioEdit against public databases (GenBank, Tremble). For 5′-RACE, we used 2 μL of 5′-RACE-ready cDNA with Universal Primer Mix (UPM, Clontech) as the forward anchor primer and with a specific reverse primer 5′RACE for SlEcR (5′-GCC GGA GGC AGA CCT AAC GC-3′) and SlUSP-5′ (5′-GTT ACG CTC CTC ACG ACA CGC G −3′). The 3′-RACE amplification was carried out with UPM as the reverse primer and a specific forward primer 3′-RACE for SlEcR (5′-TTG CGA GTA GCT CGG CGG TAT GAT GCG TC-3′) and SlUSP (5′-CGC GTG TCG TGA GGA GCG TAA C −3′). Touchdown PCRs were performed using hot start as follows: after 1 min at 94 °C, five cycles of 30 s at 94 °C and 3 min at 72 °C, then five cycles of 30 s at 94 °C, 30 s at 70 °C, and 3 min at 72 °C, then 25 cycles of 30 s at 94 °C, 30 s at 68 °C, and 3 min at 72 °C, then 10 min at 72 °C. The PCR products were gel purified (Nucleospin Extract II, Macherey–Nagel, Düren, Germany) and cloned into pCRII-TOPO plasmids (Invitrogen, Carlsbad, CA, USA). After colony isolation, plasmids were isolated by minipreps (Nucleospin Plasmid, Macherey–Nagel, Düren, Germany) and the presence of insertions was confirmed by restriction enzyme analysis. Recombinant plasmids were sequenced (GATC Biotech, Marseille, France). The full-length cDNA was generated by combining the sequences from the 5′ and 3′-RACE.