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High temperature and hexane break pupal diapause in the flesh fly, *Sarcophaga crassipalpis*, by activating ERK/MAPK

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Abstract

Pupal diapause in the flesh fly, *Sarcophaga crassipalpis*, can be terminated by exposure to high temperatures or, artificially, with a topical application of organic solvents. To analyze the molecular mechanisms involved in diapause termination we explored the possibility that the mitogen-activated protein kinases (MAPK) are involved in this response. Levels of phospho-ERK increased within 10 min after hexane application. Extracellular signal-regulated kinase (ERK) was also activated when pupae were transferred from 20 to 25 °C, thus suggesting that ERK activation is a likely component of the signal transduction pathway used to initiate development in response to diapause-terminating signals. 20-Hydroxyecdysone and cyclic GMP terminate diapause in this fly, and the juvenile hormone analog methoprene shortens the diapause, but none of these agents activated ERK. ERK was readily activated in isolated abdomens treated with hexane, thus we conclude that ERK is directly activated by the hexane treatment. ERK activation was evident in the brain, epidermis, midgut and fat body, but not in the ventral nerve mass or ring gland, thus suggesting that ERK does not act directly on the ring gland to promote ecdysteroid synthesis but exerts its effect through stimulation of the brain.
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1. Introduction

Mitogen-activated protein kinases (MAPKs) play a potential role in terminating the embryonic diapause of *Bombyx mori* (Iwata et al., 2005; Fujiwara et al., 2006a, b; Fujiwara and Shiomi, 2006) and the false melon beetle *Atrachya menetriesi* (Kidokoro et al., 2006). In both of these cases, extracellular signal-regulated kinase (ERK) is the MAPK family member that is linked to diapause termination. In this study, we explore the possibility that ERK or other MAPKs may be involved more generally in insect diapause termination by evaluating the activation (phosphorylation) of ERK, p38 MAPK and Jun N-terminal kinase (JNK) at the termination of pupal diapause in the flesh fly, *Sarcophaga crassipalpis*. In this fly, diapause can be artificially terminated with organic solvents such as hexane (Zdarek and Denlinger, 1975; Denlinger et al., 1980) or by elevation of temperature (Denlinger, 1972). We evaluate the phosphorylation response of these three MAPKs to both stimuli.

Flesh flies enter pupal diapause in response to short day length in early autumn and initiate development once again in the spring when temperatures increase (Denlinger, 1972). Like pupal diapauses in other insects (Denlinger et al., 2005), the pupal diapause in flesh flies appears to be the result of a shut-down of the brain–prothoracic gland axis (Zdarek and Denlinger, 1975). The ecdysteroid titer is low during diapause (Walker and Denlinger, 1980), and an ecdysteroid injection terminates the diapause (Zdarek and Denlinger, 1975). Juvenile hormone (JH) does not immediately break diapause (Fraenkel and Hsiao, 1968), but diapause is shortened with a JH application (Zdarek and Denlinger, 1975) and has a synergistic effect in terminating diapause when applied concurrently with ecdysteroids (Zdarek and Denlinger, 1975). The termination of diapause is also prompted by an injection of cGMP, and again the effect is synergistic when administered concurrently with ecdysteroids (Denlinger and Wingard, 1978).

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When hexane is used to terminate diapause in flesh flies the most rapid physiological response noted is a dramatic (100 ×) increase in the rate of oxygen consumption within 15 min (Denlinger et al., 1980). A brief pulse of JH activity is noted 3 h later, and the ecdysteroid titer increases 9 h after hexane application (Walker and Denlinger, 1980). Several genes are selectively upregulated (e.g. heat shock proteins) or downregulated (e.g. proliferating cell nuclear antigen) several hours after hexane application (Tammariello and Denlinger, 1998; Yocum et al., 1998; Rinehart and Denlinger, 2000; Rinehart et al., 2000). Among the most rapidly upregulated genes following hexane application are the genes encoding ecdysone receptor and its dimerization partner ultraspiracle (Rinehart et al., 2001); upregulation of both of these genes occurs within 1 h.

It remains unknown how organic solvents exert their effect on diapause termination, although it has been postulated that the solvents may affect membrane permeability of neurosecretory cells, thus promoting the release of prothoracicotropic hormone (PTTH) from the brain, which in turn stimulates ecdysteroid production in the ring gland (Denlinger et al., 1980). In this study we propose an alternative mechanism involving activation of ERK, a MAPK. We also suggest that ERK is involved as a component of signal transduction pathway that mediates the high-temperature-induced termination of diapause.

2. Materials and methods

2.1. Flies

Flies were from our established laboratory colony of *S. crassipalpis* Macquart. Non-diapausing individuals were reared under long-day conditions of 15 h light and 9 h dark at 25 °C, while diapausing pupae were produced by rearing adults under short-day conditions of 12 h light and 12 h dark at 25 °C, and larvae and pupae at 20 °C under the same short-day conditions (Denlinger, 1972). Diapausing pupae, 20–30 days after pupariation, were used for experiments unless otherwise indicated. The anterior caps of puparia were removed to observe development. To initiate diapause termination, 5 μl hexane was topically applied to the head of diapausing individuals (Denlinger et al., 1980). Injections of chemicals, in volumes of 1 μl, were made into the pupal head by means of finely drawn out glass capillaries (Zdarek and Denlinger, 1975). Tissue dissections were performed in phosphate-buffered saline (50 mM sodium phosphate, pH 7.5 and 150 mM NaCl) under a dissection microscope. For experiments utilizing ligated abdomens, the pupae were first punctured in the head to prevent rupture of the abdomen following ligation. Ligation between the head and thorax was performed using nylon thread (Yoder et al., 2006).

2.2. Chemicals

Anti-phospho-ERK, anti-ERK, anti-phospho-MAPK-ERK kinase (MEK), anti-phospho-Jun N-terminal kinase (JNK), anti-phospho-p38 MAPK, anti-biotin and anti-rabbit IgG peroxidase-conjugated goat antibodies were from Cell Signaling Technology (Beverly, MA). Each antibody was used at a dilution of 1:1000. A PhosphoPlus biotinylated protein ladder (Cell Signaling Technology) was used as a molecular marker standard and was detected by anti-biotin peroxidase-conjugated goat antibody. 20-Hydroxyecdysone, dibutyryl cAMP and 8-bromo cGMP were from Sigma-Aldrich (St. Louis, MO). Methoprene was from Chem Service (West Chester, PA). 20-Hydroxyecdysone was first dissolved in 100% ethanol, and then diluted to 5 μg/μl in 10% ethanol. Dibutyryl cAMP and 8-bromo cGMP (100 μg/μl) were dissolved in water. Methoprene was used either in its original solution (200 μg/μl) or diluted in methanol (10 μg/μl).

2.3. Western blotting

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting were described previously (Iwata et al., 2005). A single animal was used for each sample. Whole bodies or tissues were homogenized in 10 vol of SDS–PAGE buffer, boiled for 5 min, and 5 μl was applied to each lane. The gels were then transferred to polyvinylidine difluoride membranes (Immobilon-P, Millipore, Bedford, MA). Membranes were incubated with the primary antibodies, a peroxidase-conjugated secondary antibody, and visualized on X-ray films using LumiGLO chemiluminescent reagent (KPL, Gaithersburg, MD). Each treatment was replicated at least three times.

2.4. cDNA cloning

Total RNA was isolated from non-diapausing pupal brains and optic lobes of *S. crassipalpis* by homogenization in TRizol reagent (Invitrogen, Carlsbad, CA). A cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen). The ERK cDNA was amplified using degenerate PCR primers ERK1 (CAYCARACNTAYTGYCA) and ERK8 (GGRTCRTARTAYTGYTC) (where Y = T or C; R = A or G; N = A, C, G or T) using Platinum Taq DNA polymerase high fidelity (Invitrogen). The PCR conditions were described previously (Ijiro et al., 2004; Fujiwara et al., 2006a). The amplified DNA fragments were subcloned into plasmids and sequenced. The 5′ and 3′ regions of the ERK cDNA were obtained using a SMART RACE cDNA amplification kit (Clontech Laboratory, Mountain View, CA) according to the manufacturer's instructions with primers ERK16 (CTGGAGCACGATACCAGCGTGTAGCAA) and ERK9 (GCTCGTGTTGCAGATCCTGAACATGATCAC) for 5′ and 3′ RACE, respectively. Finally, the whole coding region of ERK was amplified using PCR primers ERK15 (GGAACACAACAAACGACAAAGACAA) and ERK22 (TTATACTGCTTGCTGATCGGGTTGC).

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