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Insect freeze tolerance: Roles of protein phosphatases and protein kinase A

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Abstract

Freeze-tolerant larvae of the goldenrod gall fly, *Eurosta solidaginis* Fitch, show multiple metabolic adaptations for subzero survival including the autumn synthesis of high concentrations of polyols. The induction and regulation of cold hardiness adaptations requires the intermediary action of signal transduction enzymes. The present study evaluates changes in the activities of cAMP-dependent protein kinase (PKA), protein phosphatases 1 (PP1), 2A, 2C, and protein tyrosine phosphatases (PTPs) over the course of the winter season and also in insects exposed to −4, −20 °C, or anoxic conditions in the laboratory. The increased PKA and decreased PP1 over the winter season and/or at subzero temperature support a regulatory role for these enzymes in cryoprotectant polyol synthesis. PTP activities were also strongly increased under these conditions and may act to antagonize tyrosine kinase mediated cell growth and proliferation responses and, thereby, contribute to hypometabolism and diapause over the winter.

Keywords: *Eurosta solidaginis*; Cold hardiness; Reversible protein phosphorylation; Anoxia

1. Introduction

Larvae of the goldenrod gall fly *Eurosta solidaginis* Fitch (Diptera, Tephritidae) have been studied extensively as a model of insect freeze tolerance (for review, see Baust and Nishino, 1991; Lee et al., 1996; Storey and Storey, 1991, 1992). Insect freezing survival relies on a variety of molecular adaptations that include the production of high concentrations of polyol cryoprotectants, the use of ice nucleators, metabolic rate depression (diapause) and changes in gene expression (Storey, 1997; Duman, 2001). *E. solidaginis* uses dual cryoprotectants, glycerol and sorbitol, accumulated with different seasonal patterns (Storey and Storey, 1986). The regulation of multiple enzymes involved in polyol production and energy metabolism in *E. solidaginis* has been explored (Joanisse and Storey, 1994a; Storey and Storey, 1991, 1992). A key recurring mechanism of regulation in this species and other cold-hardy insects is reversible protein phosphorylation. For example, low-temperature-triggered activation of glycogen breakdown for polyol synthesis results from phosphorylation-mediated activation of glycogen phosphorylase (GP) (Hayakawa, 1985), whereas the inhibition of gluconeogenesis that prevents back-flow of carbon is mediated by phosphorylation inhibition of glycogen synthetase (GS) and dephosphorylation inhibition of fructose-1,6-bisphosphatase (Muise and Storey, 1997, 1999). Changes in gene expression are also typically mediated by signal transduction cascades involving protein kinases and phosphatases. A variety of genes that are cold- or freeze-responsive are now known for *E. solidaginis* including the hypoxia-inducible transcription factor (*hif-1*) (Morin et al., 2005).

The regulation of polyol synthesis in insects has long been linked with differential cold-responsiveness of glycogen phosphorylase kinase (GPK) and protein phosphatase 1 (PP1) in the control of GP (Hayakawa, 1985) but roles

Abbreviations: cAMP, adenosine 3′, 5′-cyclic monophosphate; EGF, epidermal growth factor; GP, glycogen phosphorylase; GS, glycogen synthetase; PKA, cyclic AMP-dependent protein kinase; PKAc, active catalytic subunit of PKA; PMSF, phenylmethylsulfonyl fluoride; PP, serine/threonine protein phosphatase; PTP, protein tyrosine phosphatase

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for other protein kinases and protein phosphatases in insect cold hardiness have not been explored. This study focuses primarily on the responses of protein phosphatases in *E. solidaginis* analyzing seasonal changes in enzyme activities, responses to cold and freeze/thaw exposures, and the effects of anoxia exposure on PP1, other serine/threonine protein phosphatases 2A and 2C (PP2A, PP2C), and protein tyrosine phosphatases (PTP). The responses of protein kinase A (PKA) are also analyzed.

2. Materials and methods

2.1. Chemicals

Radioactive [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from New England Nuclear (Montreal, PQ). Peptides for PKA, PP2 and PTP assay were synthesized by the Protein CORE facility, Queen's University (Kingston, ON). Okadaic acid was from CalBioChem (La Jolla, CA) and other chemicals and chromatography materials were obtained from Fisher Scientific (Ottawa, ON) or Sigma Chemical Company (St. Louis, MO).

2.2. Animals

Stem galls containing mature larvae of *E. solidaginis* were collected from goldenrod plants (*Solidago* sp.) during September in Ottawa, Ontario. Some galls were held outdoors in cloth bags and sampled about once a month between September and April. For sampling, galls were rapidly opened and living insects (assessed by visual inspection) were immediately removed, frozen in liquid nitrogen and then stored at −80 °C. Others galls were placed in lab incubators at 15 °C for acclimation. Control larvae were sampled at the end of a 2 week acclimation at 15 °C. Two groups of insects were then cooled to −4 °C for 24 h; one group was sampled, whereas the other group was returned to 15 °C and sampled 48 h later. Other groups of insects were acclimated at 4 °C for 2 weeks and then transferred to −20 °C; one group was sampled after 24 h at −20 °C, whereas the rest were returned to 15 °C and sampled after 2, 4, 8, 12 or 24 h. For anoxia studies, 15 °C-acclimated larvae were removed from their galls and placed in three Petri plates that were returned to 15 °C. Larvae on one plate were sampled as aerobic controls. Two plates of larvae were given anoxia exposure at 15 °C by placing the plates in a container through which 100% nitrogen gas was flushed for 30 min. The container was then sealed. One plate was sampled at the end of 24 h anoxic exposure, whereas the larvae on the other plate were returned to aerated conditions to recover at 15 °C for 48 h before sampling.

2.3. PKA tissue extraction and assay

Whole frozen insects were rapidly weighed and homogenized 1:10 (w/v), with a few crystals of phenylmethylsulfonyl fluoride (PMSF) added, using a Prohomogenizer 200 in ice-cold 20 mM potassium phosphate, pH 6.8, 2 mM EDTA, 15 mM ß-mercaptoethanol. The homogenate was centrifuged at 13,000*g* for 3 min at 5 °C and the supernatant was removed and stored on ice until use. PKA was assayed by measuring the incorporation of ^{32}P from ^{32}P-ATP onto Kemptide (LRRASLG), a synthetic phosphate-accepting peptide, in the presence (for total PKA activity) versus absence (for activity of the free catalytic subunit, PKAc) of 0.1 mM adenosine 3′,5′-cyclic monophosphate, as described by Pfister and Storey (2002a). One unit of activity is defined as the amount of enzyme that catalyzed the incorporation of 1 nmol ^{32}P onto the substrate per minute at 23 °C.

2.4. PP1 extraction and assay

Whole frozen insects were rapidly weighed and homogenized 1:3 (w/v) using a Prohomogenizer 200 and ice-cold buffer A (20 mM Tris–HCl, pH 7.4, 2 mM EDTA, 2 mM EGTA, 15 mM ß-mercaptoethanol) containing the protease inhibitors: 1 mM PMSF, 0.1 mM TPCK, 1 µg/ml aprotinin and 5 mM benzamidine. Homogenates were centrifuged in a BioTek microfuge at 1000*g* for 3 min at 5 °C. Supernatants were removed and assayed immediately for active PP1. Assays of concentrated extracts give estimates of PP1 activities at physiological levels of modulating proteins and other factors (Toth et al., 1988). Aliquots of extracts were also diluted 1:20 v/v in buffer A containing 2 mg/ml bovine serum albumin to stimulate dissociation of modulators and were then used to measure "total" PP1 activity (Drake and Palmer, 1995); initial tests showed that this dilution gave maximal PP1 activity (data not shown). PP1 activity was measured at 23 °C by monitoring ^{32}P cleavage from ^{32}P-labeled phosphorylase, as described by Pfister and Storey (2002b). One unit of activity is defined as the amount of enzyme that releases 1 nmol of phosphate per minute at 23 °C.

2.5. PP2 extraction and assay

Extracts of whole frozen insects were prepared as for PP1 except for a 1:10 (w/v) dilution. Homogenates were centrifuged at 13,000*g* for 20 min, and the supernatant was removed and desalted by low-speed centrifugation (1 min at room temperature) through 5 ml columns of Sephadex G-25 equilibrated in Buffer A. The eluant was removed, passed through a second, fresh column and then held on ice until use. Assays for PP2A and PP2C were as previously described (Cowan et al., 2000). Briefly, the assay mixture for PP2A contained 150 µM peptide RRA(**pT**)VA, 0.2 mM EGTA, 0.02% ß-mercaptoethanol, and 50 mM imidazole, pH 7.2. PP2A activity was detected as the difference in activity in the absence versus presence (blank) of 2.5 nM okadaic acid. Reactions were started by adding 10 µl enzyme extract (final assay volume = 50 µl) and then incubated for 40 min. Reactions were terminated with the addition of 50 µl