ISJ 5: 1-11, 2008 ISSN 1824-307X

REVIEW

Nongenomic and genomic actions of an insect steroid coordinately regulate programmed cell death of anterior silk glands of *Bombyx mori*

M Manaboon, M Iga, S Sakurai

Division of Life Sciences, Graduate School of Natural Science and Technology, Kanazawa University, Japan

Accepted February 4, 2008

Abstract

The insect steroid hormone 20-hydroxyecdysone (20E) induces programmed cell death of larva-specific tissues at pupal metamorphosis. In the silkworm *Bombyx mori*, the anterior silk gland undergoes cell death in response to the metamorphic peak titer of ecdysteroids *in vivo* and also to 20E *in vitro*. Although 20E elicits early gene activation, an additional 20E stimulus is required for completion of cell death. This additional stimulus involves caspase-3-like protease activation, indicating that 20E also acts through a nongenomic mechanism. Studies using various inhibitors, agonists, and antagonists have shown that cell condensation is under the control of 20E genomic action, and that 20E nongenomic action begins with 20E binding to the putative membrane-bound ecdysone receptor, which is probably a G-protein-coupled receptor. This step is followed by a signaling pathway comprising phospholipase C/inositol 3,4,5-triphosphate/Ca^{2+}/protein kinase C/caspase-3-like protease, which induces DNA and nuclear fragmentation. Nuclear condensation is regulated by signaling of calmodulin/calmodulin-dependent protein kinase II (CaMKII), but CaMKII activation is independent of intracellular Ca^{2+} elevation. In addition, the genomic action of 20E is indispensable for driving its nongenomic action, indicating that crosstalk between genomic and nongenomic action plays a significant role in 20E-induced cell death.

Key words: ecdysone; programmed cell death; nongenomic; genomic; membrane ecdysone receptor; calcium; protein kinases

Introduction

Steroid hormones regulate development, reproduction, metabolism, and homeostasis in insects and mammals. In vertebrates, steroids including estrogens, androgens, progesterone and glucocorticoids control cell death (Herold *et al.*, 2006), and in insects, the steroid 20-hydroxyecdysone (20E) induces apoptosis (Terashima *et al.*, 2000; Fahrbach *et al.*, 2005). Regulation of those steroids has been studied primarily in conjunction with steroid receptor function.

Steroids regulate cell death by two major mechanisms. Some, like estrogens, act as survival factors that trigger cell death when withdrawn, while others, such as glucocorticoids and 20E, actively trigger cell death. Although little is known about the mode of action of steroids in cell death, recent studies have provided insight into the genetic mechanisms underlying 20E-induced programmed cell death in the salivary glands of *Drosophila melanogaster*.

It is well understood that steroid hormones regulate gene expression by binding to a nuclear receptor. Thus, this genetic mechanism has been the major topic of study in relation to cell death. The genetic aspects, however, do not sufficiently account for the molecular mechanisms underlying steroid-induced cell death, which is accompanied at the later stages by caspase-3 activation (Woo *et al.*, 1998). Parts of this pathway are also used in ligand-dependent cell death, such as that observed for Fas-ligand-dependent cell death in lymphocytes (Winoto and Littman, 2002). Similarly, 20E-induced cell death in *Drosophila* salivary glands includes serial activation/inhibition of a protease cascade, including DIAP1/DRONC (a homologue of caspase

Corresponding author:
Sho Sakurai, Ph.D.
Division of Life Sciences
Graduate School of Natural Science and Technology
Kanazawa University
Kakumamachi, Kanazawa 920-1192, Japan
e-mail: ssakurai@kenroku.kanazawa-u.ac.jp

9) pathway leading executioner caspase activation such as drICE and DECAY (Dorstyn *et al*., 1999; Yu *et al*., 2002; Leulier *et al*., 2006; Callus and Vaux, 2007). Both the genomic pathway that regulates early genes, leading to activation of death genes (Yin and Thummel, 2005), and the nongenomic pathway that activates caspase-3, are required for successful execution of programmed cell death (Martin and Baehrecke, 2004); however, little is known about how the two pathways are linked to each other.

Regulation of programmed cell death by 20E

Post-embryonic development of insects is associated with several molting cycles. In holometabolous insects, the larvae undergo larval-pupal ecdysis after growth. *Bombyx mori* (silkworm) and *D. melanogaster* undergo four and two larval-larval molts, respectively. Pupal ecdysis is accompanied by various developmental changes in tissues at the cellular and molecular levels (Riddiford, 1994; Henrich *et al*., 1999). Larval-pupal transformation begins at the end of the penultimate instar (fourth larval instar in *Bombyx*), when wing imaginal discs and leg primordia are committed to undergoing pupal metamorphosis at the following ecdysis (Obara *et al*., 2002; Koyama and Sakurai, unpublished). In the feeding period of the last larval instar (fifth instar), wing discs and epidermal cells are pupally committed (Riddiford, 1996; Obara *et al*., 2002), while silk glands are committed to die after pupal ecdysis (Kakei *et al*., 2005). All of these events are under the control of a single steroid hormone, 20E.

Silk glands, which are the largest tissues in *Bombyx* last instar larvae, degenerate soon after pupation through 20E-induced programmed cell death. At the end of the feeding stage, the ecdysteroid concentration in the hemolymph increases slightly to a small peak known as the "commitment peak". This rise causes feeding to cease and induces spinning of silk thread from the silk glands.

Silk glands consist of three parts: the anterior, middle, and posterior silk glands. The middle silk gland produces sericin, the "glue" protein, and the posterior silk gland produces fibroin, the silk thread protein itself. In the anterior silk gland (ASG), the middle and posterior glands join to form a duct where the silk thread is spun by coating of the amorphous fibroin protein with sericin. The ASG is entirely covered with a thick basement lamina and lined with a thin cuticle layer (cuticular intima) on the lumen-side surface (Akai, 1983). It consists of hundreds of a single type of cell, which is rather flat and irregularly hexagonal and possesses highly branched, thread-like nuclei (Fig. 1).

The ASG undergoes programmed cell death in response to a large metamorphic peak of ecdysteroid in the hemolymph. Detachment at the cell boundary is the first morphological change (Chinzei, 1975; Terashima *et al*., 2000) and occurs on the third day (G2) of the prepupal period that begins with gut purge and lasts for 3-4 days. Then, the cells condense and become round in shape (cell condensation), and the nuclear branches thicken (nuclear condensation). This step is followed by DNA fragmentation, which is detectable as a ladder pattern on agarose gel electrophoresis, and by nuclear fragmentation (Iga *et al*., 2007). Finally, small granules containing the fragmented DNA, which probably correspond to apoptotic bodies, are formed (Fig. 2). The cell death process begins in response to the small ecdysteroid peak at the molecular level and is fully activated by the metamorphic peak, when the cells are slightly rounded in shape. Further 20E stimulus is not needed after this point for cell death, ending with apoptotic body formation (Terashima *et al*., 2000).

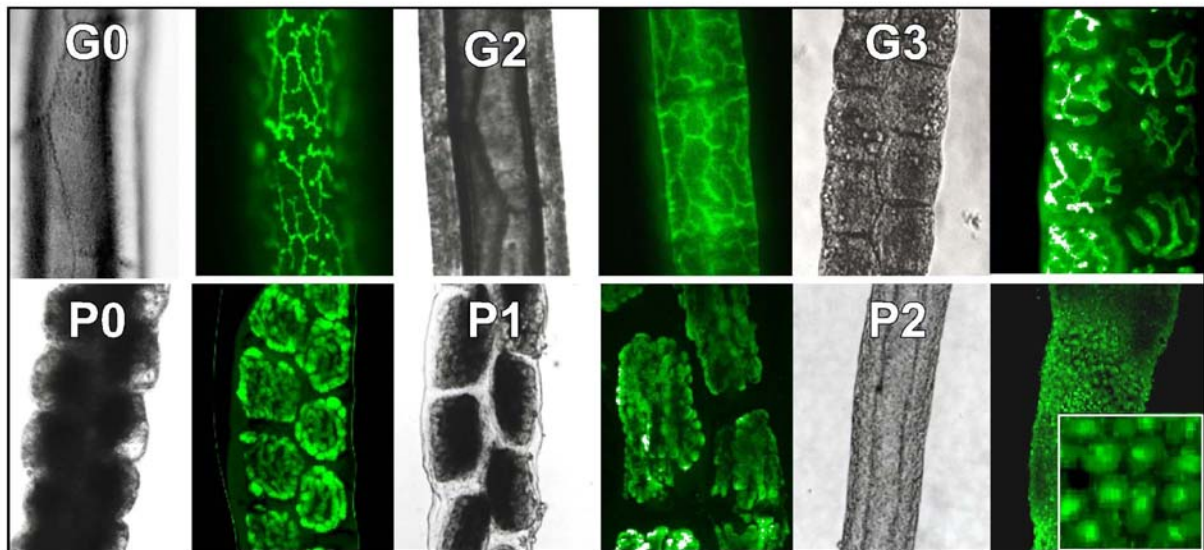
Although the *Drosophila* salivary gland is not a homologue of the silk gland, its cells do undergo cell death in response to a metamorphic ecdysteroid peak in the prepupal period. *Bombyx* larvae have salivary glands that survive until adult stage. These glands produce cocoonase, which opens a window in the cocoon at eclosion through which the adult escapes (Kafatos *et al*., 1967). The salivary gland cells of *Drosophila* are small and have round nuclei, which is disadvantageous for following changes in cellular and nuclear morphologies. Hence, morphological descriptions of cell death in these glands have been focused on DNA fragmentation as indicated by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) method (Daish *et al*., 2004), since the DNA is difficult to recover in sufficient quantity to observe the ladder pattern on a gel (see Martin and Baehrecke, 2004).

Genetic regulation of 20E-induced programmed cell death in *Drosophila* salivary glands has been the subject of extensive study. However, only the pathway leading to DNA fragmentation has been studied. In contrast, the flat shape and highly branched nuclei of ASG cells are conducive to the following changes in cellular and nuclear morphology and allow discrimination of the pathways leading to changes in cell morphology, nuclear condensation, DNA fragmentation, nuclear fragmentation, and apoptotic body formation (Fig. 1).

EcR isoform involved in programmed cell death

The nuclear receptor for 20E is a heterodimer of the ecdysone receptor, EcR, and its partner protein, Ultraspiracle (Usp) (Yao *et al*., 1992; Henrich *et al*., 1999). Binding of 20E to its receptor regulates the expression of early genes and then of effecter genes (Yin and Thummel, 2005). The genomic effects of 20E action, beginning with serial activation/inhibition of gene expression, have been studied extensively using *Drosophila* salivary glands (Lee and Baehrecke, 2001; Baehrecke, 2003; Yin and Thummel, 2005), which are larva-specific. At the time of pupal metamorphosis, these glands undergo cell death in response to 20E, which induces pupariation and then pupation. The hierarchical control of expression of early genes in the salivary glands begins with 20E activation of *EcR-B1*, which encodes an EcR isoform involved in cell death. In *Drosophila*, knockout of individual EcR isoforms has shown that mutations in *EcR-A* arrest development at the end of the last (third) larval instar

(A) PCD progression in vivo



(B) PCD progression in vitro

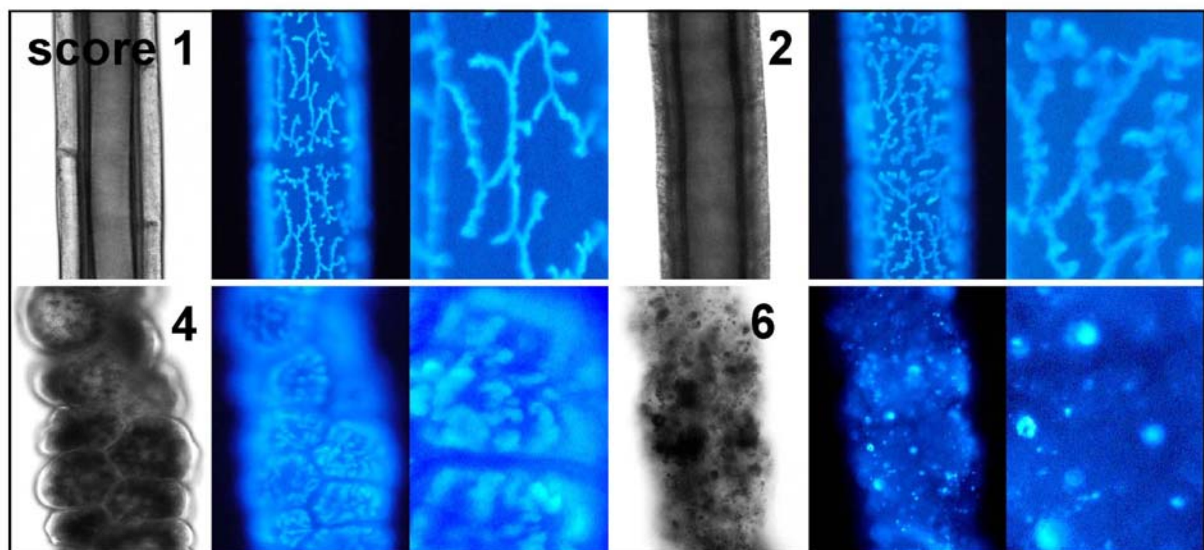


Fig. 1 (A) *In vivo* progression of programmed cell death of *Bombyx* anterior silk glands (ASGs) after gut purge. G0-G3, 0-3 days after gut purge; P0-P2, 0-2 days after pupation. For each pair of panels representing an individual day, images were obtained by light microscopy (left panel) and by fluorescence microscopy after staining with acridine orange (right panel). Note that the G0 cells are hexagonal in shape with finely branched nuclei. From G3 to P1, cellular and nuclear condensation occurs. Finally, in P2, numerous granules are formed. Inset: enlargement of acridine orange-stained image showing the small granules. At P2, the ASGs are lined with thick basal membranes, which serve to maintain the outer shape, but soon after P2, the ASGs degenerate completely and disappear from the pupal body. **(B)** *In vitro* progression of programmed cell death. ASGs were cultured with 20E (0.5 μg/ml) and stained with DAPI. In the far right panels for individual scores, bottom row of panels, the DAPI-stained images are enlarged to show the shape of the nuclei. See Fig. 2 for detailed progression.

and prevent pupation, with the tissues that normally form adult structures remaining in their larval forms (Davis *et al*., 2005). *EcR-B1* mRNA predominates in tissues (including salivary glands) destined to undergo programmed cell death at pupal metamorphosis (Talbot *et al*., 1993). In an *EcR-B1* loss-of-function *Drosophila* mutant, the ecdysone-inducible genes in the larval salivary glands failed to activate (Bender *et al*., 1997). Furthermore, an *EcR-B1* knockout mutation prevents programmed cell death

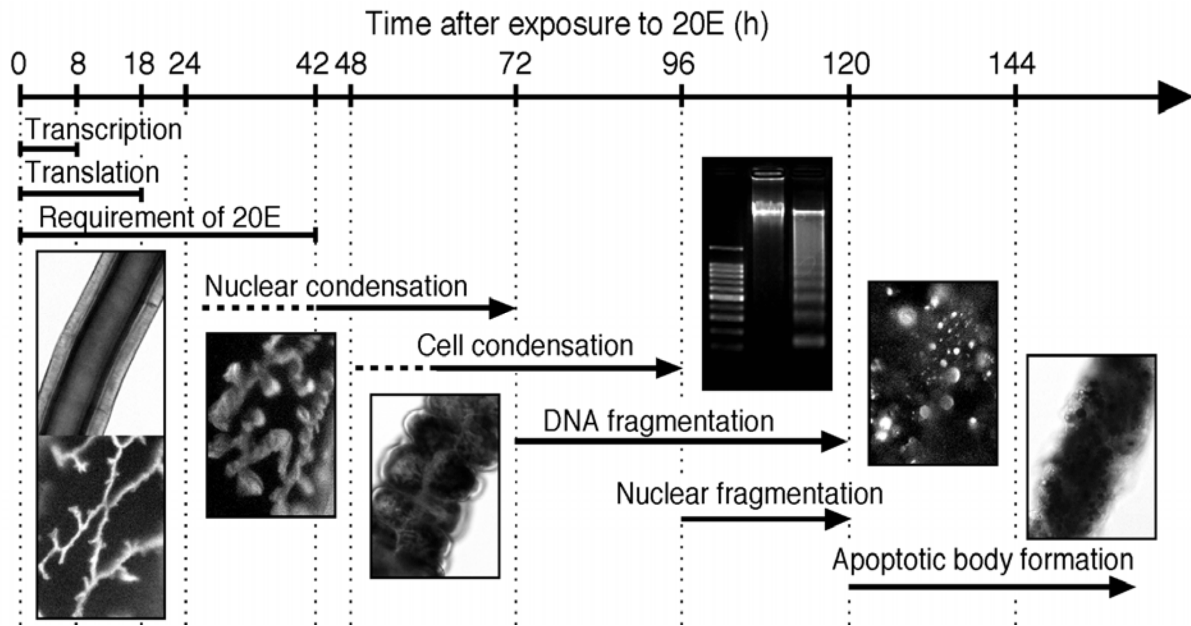


Fig. 2 Death sequence *in vitro*. Solid lines indicate the time period during which individual cellular events occur (see Terashima *et al*., 2000 and Iga *et al*., 2007 for details). Modified from Iga *et al*. (2007).

in pupal bodies but does not affect the larval-pupal transformation. Thus, EcR-B1 is the EcR isoform involved in metamorphic changes in larva-specific tissues that undergo cell death.

In insect species other than *Drosophila*, the roles of the EcR isoforms appear to be reversed. Swallowtail butterflies commonly possess wings with intricately "cut" edges, especially in the posterior region of the hind wings. Immediately after elongation of the wing imaginal discs at pupation, the wing edge is smooth. The swallowtail shape is then formed by elimination of the outer regions of the pupal wings by programmed cell death (Suyama *et al*., 2003). In the pupal wings of the swallowtail *Papilio xuthus*, two EcR isoforms are expressed: *EcR-B1* is expressed exclusively in the proximal part of the margins between the cells that survive and form adult wings and the cells that undergo cell death, while *EcR-A* is expressed in the distal region that is eliminated in response to 20E. Thus, EcR-A is the EcR isoform involved in region-specific programmed cell death in the pupal wings of *P. xuthus*. Similarly, in *Blattella germanica* (cockroach), EcR-A mediates programmed cell death of the prothoracic glands (Cruz *et al*., 2006). In *Bombyx* ASGs, *EcR-A* is induced at the beginning of the prepupal period, and its mRNA level increases until day 2 of the prepupal period (Kamimura *et al*., 1997; Sekimoto *et al*., 2006). In other tissues, such as wing discs, epidermis and midguts that do not undergo cell death, *EcR-B1* is the predominant EcR isoform (Kamimura *et al*., 1997). Accordingly, although there is no knockout or knockdown data in lepidopterans, *EcR-A* is likely to be the isoform responsible for induction of programmed cell death in lepidopteran insects.

Genetic hierarchies downstream of EcR

Genetic regulation downstream of EcR is well documented in *Drosophila* salivary glands (Jiang *et al*., 2000; Yin and Thummel, 2005). The genes thus far shown to be involved in programmed cell death are the early genes *EcR*, *broad complex* (*BR-C*), *E74*, *E75*, and *E93*; the late gene β*FTZ-F1* (the *Drosophila* homologue of *fushitarazu*); the death activator genes reaper (*rpr*) and head involution (*hid*); and *dronc* and *drice*, which are involved in the pathway leading to caspase activation (Jiang *et al*., 2000; Yin and Thummel, 2005, 2007). There are two ecdysteroid surges during the *Drosophila* third instar period, as in *Bombyx* (Sakurai *et al*., 1998); the first surge induces pupariation, and the second, large surge induces pupation. The surge that begins shortly before pupariation induces *BR-C*, *E74A*, and *E75A*, but not the death activators, *rpr* and *hid*. The genes *E75A*, *E75B*, *E74A*, and *BR-C* are upregulated in response to the second ecdysone surge, leading to death activator gene induction (Jiang *et al*., 2000; Dubrovsky, 2005; Yin and Thummel, 2005).

In *E93*, and *BR-C* mutants, the salivary glands fail to undergo cell death in response to 20E. This dysfunction is probably due to the lack of active caspase-3/drICE, as indicated by reduction of *drice* expression in *E74* mutant salivary glands (Lee *et al*., 2003) and by suppression of DNA/nuclear fragmentation (Martin and Baehrecke, 2004). These data provide further support for an interaction between genomic and nongenomic actions of 20E.

Direct evidence for early gene regulation has also been found in *B. germanica* (Cruz *et al*., 2006, 2007). Knockdown of *B. germanica* hormone

receptor 3 (*BgHR3*) by a single injection of double-stranded RNA into early last (sixth) instar *B. germanica* nymphs induces an arrest of developmental to adult, showing that *BgHR3* is involved in hierarchical stimulation of the early genes. *BgEcR-A* knockdown by RNA interference prevents programmed cell death of the prothoracic glands, which normally occurs after adult eclosion, at the end of the same instar. It also markedly reduces the *BgHR3* level in the glands, indicating an involvement of *BgHR3* in 20E-induced programmed cell death. *E75* knockdown induces premature degeneration of the prothoracic glands, indicating that E75 acts as a "suppressor" of programmed cell death (Mane-Padros *et al*., 2008). Therefore, in *B. germanica*, 20E stimulates the EcR-A/BgHR3 pathway to activate the death activator genes, while also suppressing *E75* expression in the prothoracic glands. This mechanism is similar to that found in *Drosophila* salivary glands, where *E75* is required to repress the death inhibitor gene *diap2* (Jiang *et al*., 2000; Palanker *et al*., 2006). In *Bombyx* ASGs, the temporal profiles of *E75* and *Bombyx* hormone receptor 3 (*BHR3*) gene expression after 20E challenge (Sekimoto *et al*., 2006) are very similar to those in *Drosophila* salivary glands (Lam *et al*., 1999), an indication for the presence of a similar transcription hierarchical control to *B. germanica* prothoracic glands and *Drosophila* salivary glands.

Gene expression profiling has provided insight into the early gene regulation of 20E-induced cell death of *Bombyx* ASGs. The developmental expression profiles of early and early-late genes indicate that early gene regulation in *Bombyx* is similar to that in *Drosophila* (Jiang *et al*., 2000; Sekimoto *et al*., 2006, 2007). The times at which upregulation begins and expression peaks during the feeding and prepupal periods of *Bombyx* are very similar to those in the *Drosophila* prepupal period, with two exceptions described below (Sekimoto *et al*., 2006)

In *Drosophila*, there are two peaks in the hemolymph ecdysteroid titer, one for pupariation and the other for pupation (Riddiford, 1996). In *Bombyx*, there are also two peaks, one for entering the prepupal period and the other for pupation. However, the two peaks are not separated by a distinct time interval; rather, the titer gradually increases with substantial fluctuations from the rise of the small peak to the maximum peak titer (Sakurai *et al*., 1998).

Nongenomic action of vertebrate steroid hormones

The various tissues and cells of vertebrates respond rapidly to steroid hormones (Wehling, 1997; Lösel and Wehling, 2003; Lösel *et al*., 2003; Sergeev, 2005; Tasker *et al*., 2006). On the other hand, the genomic effects of steroid hormones are not fully realized for several hours or days. The rapid responses are mediated by ligand-dependent channel proteins in the plasma membrane (Qiu *et al*., 2006), by nuclear receptors localized to the plasma membrane (Simoncini *et al*., 2002, 2004), or by membrane-bound ligand receptors (Revankar *et al*., 2005; Boonyaratanakornkit and Edward, 2007).

Although the nongenomic actions of glucocorticoids have been well elucidated (Herr *et al*., 2007), the identity of the glucocorticoid receptor is, as yet, unknown. It is assumed to be a G-protein coupled receptor (GPCR). Studies of the novel mechanisms by which glucocorticoids suppress the immune response of T-lymphocytes have suggested that glucocorticoids have rapid effects on trans-membrane currents, the T-cell receptor complex, and mitogen-activated protein (MAP) kinase signaling pathways, in addition to elevating the level of intracellular Ca^{2+} (Löwenberg *et al*., 2007).

The steroid compound 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP) acts on the sea trout oocyte to stimulate ovarian maturation (Zhu *et al*., 2003). It brings about a decrease in the level of cAMP *via* binding to the membrane-bound progestin receptor (mPR), the first steroid membrane receptor identified as a GPCR (Zhu *et al*., 2003; Thomas *et al*., 2007). By coupling to G-protein αi subunit, mPR inhibits adenylyl cyclase, thus decreasing the intracellular cAMP level (Pace and Thomas, 2005). Unlike other steroids with specific nuclear receptors, 17α,20β-DP does not have a nuclear receptor. Therefore, it must elicit its effects only through nongenomic action.

The nongenomic action of estrogen is mediated by a membrane-bound receptor or by an alternative pathway initiating with the cytoplasmic estrogen receptor. Binding of estrogen to the membrane-bound estrogen receptor (GPR30) increases intracellular cAMP levels by activating adenylyl cyclase (Lösel *et al*., 2003; Rønnekleiv and Kelly, 2005; Thomas *et al*., 2005; Zivadinovic *et al*., 2005). Estrogen also binds with estrogen receptor α in the cytoplasm and activates phosphatidylinositol 3-kinase (PI3K), thus activating a protein kinase cascade (Simoncini *et al*., 2002).

In insects, immunohistochemical analysis in *Rhodnius prolixus* (Schlattner *et al*., 2006) and *Bombyx* (Hossain *et al*., 2006) has identified an ecdysone receptor in the cytoplasm beneath the plasma membrane of brain neurosecretory cells, suggesting that EcR is localized close to the plasma membrane of other tissue cells, and therefore would be involved in ASG cell death. However, inhibitors of PI3K and the MAP kinase/extracellular signal-regulated kinase (ERK) kinase do not interfere with the 20E-triggered death sequence (Iga *et al*., 2007; Iga and Sakurai, unpublished). Thus, 20E-induced programmed cell death might not involve the EcR/PI3K pathway, although this pathway may be present in insect cells.

Possible nongenomic action of 20E

Arthropods respond rapidly to 20E, which is believed to affect Na^+-K^+ ATPase, Na^+-H^+ exchangers, K^+ channels, ecdysone transport, electrolyte (Na^+, K^+, H^+) transport, and second messenger (cAMP, Ca^{2+}) levels, and acts as a neuromodulator (see Tomaschko, 1999 for review). In the wing imaginal discs of *Hyalophora gloveri* (giant silk moth), 20E increases cAMP levels (Applebaum and Gilbert, 1972), indicating a

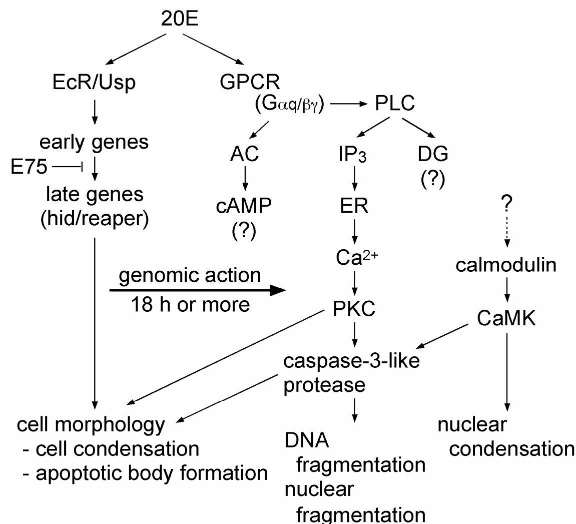


Fig. 3 Possible interaction between genomic and nongenomic actions of 20E. See text for details. Modified from Iga *et al*. (2007).

nongenomic activity of 20E. It stimulates NO-mediated cell proliferation in the pupal eye of *Manduca sexta* (tobacco hornworm) (Champlin and Truman, 1998, 2000). The NO-mediated action of 20E is thought to involve activation of the nuclear EcR, which upregulates the gene for NO synthase (NOS), thereby increasing the activity of the enzyme.

In the wing imaginal discs of *Bombyx* fifth instar larvae, 20E stimulates cell proliferation (Koyama *et al*., 2003). The presence of α-amanitin, a transcription inhibitor, does not affect this action of 20E (Koyama and Sakurai, unpublished data), indicating that *de novo* gene expression is unnecessary. Thus, 20E probably elicits its effect on cell proliferation through a nongenomic pathway, like the estrogen/ERα/PI3K/Akt/NOS pathway (Simoncini *et al*., 2002).

The molecular mechanisms that stimulate the 20E nongenomic pathway are largely unknown, but in *Drosophila*, stimulation of this pathway is known to involve the catecholamine receptor (dopamine/ecdysone receptor; DmDopEcR) (Srivastava *et al*., 2005). DmDopEcR is a GPCR that binds 20E and dopamine in different binding pockets; binding of 20E to its pocket on DmDopEcR elevates the intracellular cAMP level. Although DmDopEcR acts as a 20E membrane receptor, it does not appear to be the membrane receptor involved in 20E-induced programmed cell death, since neither cAMP nor protein kinase A mediates 20E signaling up to ASG cell death (Iga *et al*., 2007).

In the sections that follow below, I will present some details of the programmed cell death of *Bombyx* ASGs and discuss the 20E signaling pathway. Because our information concerning 20E signaling derives entirely from our own studies, the following discussion is based on our unpublished data, except where a reference is cited (see Fig. 3 for the following sections).

Involvement of the ecdysone membrane receptor in completion of cell death

Exposure to 20E induces programmed cell death of *Bombyx* ASGs. Apoptotic body formation occurs 120 to 144 h after 20E challenge (Fig. 1), but timed additions of α-amanitin have shown that *de novo* expression of the genes needed for execution of cell death is complete within 8 h of challenge (Terashima *et al*., 2000). Similarly, studies using cycloheximide, a translation inhibitor, have shown that protein synthesis is completed within 18 h of 20E challenge (Terashima *et al*., 2000). In the classical model for steroid hormone action through nuclear receptor binding, these data would suggest that, after 18 h of challenge, the 20E stimulus is no longer needed for the cell death to occur. Nevertheless, cell death is not fully realized unless 20E is present continuously for 42 h (Terashima *et al*., 2000), suggesting that 20E activates a nongenomic pathway in addition to exerting its genomic action (Fig. 2).

Since activation of the genetic pathway required for programmed cell death of *Bombyx* ASGs is complete within 18 h of the 20E challenge (Fig. 2), we examined the nongenomic action of 20E using ASGs that had been incubated with 20E for 18 h. Under these culture conditions, 20E increased the intracellular level of cAMP (Elmogy *et al*., 2006), suggesting the presence of an ecdysone membrane receptor (mEcR; Elmogy *et al*., 2004, 2007). [Subsequently, cell death was found not to involve cAMP (Iga *et al*., 2007).]

The plasma membrane fraction prepared from the pre-cultured ASGs bound to ponasterone A, a plant ecdysteroid with a K_m of 18 nM (Elmogy *et al*., 2004), a value being comparable to the K_m for DmDopEcR (Srivastava *et al*., 2005). This value is approximately 10 times higher than the K_m for the lepidopteran EcR/Usp complex (Minakuchi *et al*., 2002) The binding protein(s) in *Bombyx* ASGs are integral plasma membrane proteins (Elmogy *et al*., 2004, 2007). In addition, the bisacrylkydrazine ecdysone agonists, which bind with a high affinity to EcR/Usp, exhibit very low affinities to the putative mEcR and also DmDopEcR, showing that the mEcR is not the classical EcR. These biochemical and topological evidence indicates the presence of mEcR in *Bombyx* ASGs.

Calcium mobilization and inositol triphosphate

Agonists, antagonists, and inhibitors are powerful tools for studying signal transduction pathways. Since serial activation of protein kinases is a major feature of these pathways, we used various kinase inhibitors to search for the kinase involved in 20E signaling. Inhibition of protein kinase C (PKC) suppressed the progression of death sequence except for nuclear condensation, suggesting that Ca^{2+} is the second messenger. In fact, a Ca^{2+} ionophore added to the ASG culture after 18 h of 20E challenge mimicked the DNA and nuclear fragmentation-inducing effects of 20E. When the ionophore was added at the beginning of the culture, however, it elicited no change in the ASGs, indicating that the genomic effects of 20E are

a prerequisite for driving the Ca^{2+} pathway (Iga *et al.*, 2007).

Verapamil, which blocks voltage-activated Ca^{2+} channels, had no effect on the death sequence, indicating that 20E does not activate L-type Ca^{2+} channels (Lieberherr and Grosse, 1994). Flunarizine, which blocks ligand-dependent (T-type) Ca^{2+} channels (Qui *et al.*, 2006), inhibited DNA and nuclear fragmentation, but allowed other cellular responses (cell and nuclear condensation) to occur normally. The inhibitory effect of flunarizine indicates that the mEcR could be a L-type Ca^{2+} channel-coupled receptor (Manaboon and Sakurai, unpublished). In this case, the Ca^{2+} influx could come from the medium. However, 20E induced complete cell death in Ca^{2+}-free medium, and flunarizine exhibited the same inhibitory effects in Ca^{2+}-free medium as in normal medium. Thus, the Ca^{2+} reservoir must be inside the cell, most likely in the endoplasmic reticulum (ER), which provides Ca^{2+} for many signal transduction pathways. In fact, 2-aminoethyl diphenylborinate, an inhibitor of the inositol 3,4,5-triphosphate receptor (IP_3R) on the ER membrane inhibits DNA and nuclear fragmentation (Manaboon and Sakurai, unpublished), consistent with the ER being the reservoir for intracellular Ca^{2+} elevation. Flunarizine may not act as a Ca^{2+} channel blocker in *Bombyx* ASGs but inhibit calmodulin/CaMKII pathway, which may mediate the 20E-induced DNA and nuclear fragmentations (see below), as demonstrated in bovine brains (Kubo *et al.*, 1984).

Upstream of Ca^{2+}

Suramin, an inhibitor of G-proteins, suppresses the cellular responses to 20E (DNA and nuclear fragmentation) underlying the nongenomic pathway. U73122, an inhibitor of phospholipase C (PLC), also inhibits these responses, indicating that the G-protein αq subunit ($G_{\alpha q}$)/PLC/IP_3 pathway acts downstream of the mEcR (Manaboon and Sakurai, unpublished).

In the prepupal period, the intracellular cAMP level increases transiently at the third day of gut purge, when the ASGs are fully stimulated to complete programmed cell death with no further 20E stimulus (Elmogy *et al.*, 2007). In ASGs cultured with 20E, intracellular cAMP also increases, beginning 24 h after 20E challenge, indicating an involvement of G-protein αs subunit ($G_{\alpha s}$). However, dibutyl cAMP, a membrane-permeable cAMP analogue, does not mimic 20E action, and an inhibitor of protein kinase A (PKA) has no effect on 20E signaling (Iga *et al.*, 2007), demonstrating cAMP/PKA is not involved in induction of cell death. In Chinese hamster ovary cells expressing histamine H1 receptor, histamine increases intracellular cAMP levels through activation of the receptor, which releases G-protein $\beta\gamma$ subunits ($G_{\beta\gamma}$) that activate adenylyl cyclase (Maruko *et al.*, 2005). If $G_{\beta\gamma}$/adenylyl cyclase pathway occur in the ASGs, the mEcR may be coupled with both $G_{\alpha q}$, but the role of $G_{\beta\gamma}$ and diacylglyceride in cell death remains to be seen.

Downstream of Ca^{2+}

As described above, inhibition of PKC suppresses DNA and nuclear fragmentation. PKC inhibitor suppresses these cellular responses when added 24 h, but not 48 h, after 20E challenge, showing that the PKC activation required for inducing these responses is complete by 48 h. However, when PKC activity was assessed using a fluorescence labeled PKC substrate, it was shown to be at substantial levels with small fluctuations (Iga *et al.*, 2007). Since the substrate is phosphorylated by a broad spectrum of PKC isozymes, the isozyme involved in 20E signaling remains to be identified. In rat, phorbol esters increase intracellular anti-apoptotic protein, phosphoprotein enriched in diabetes/phosphoprotein enriched in astrocytes (PED/PEA)-15 by reducing its proteasomal degradation, thereby enhancing its anti-apoptotic action. PKC-ζ and calmodulin/calmodulin-dependent protein kinase II (CaMKII) activities are necessary for phorbol ester-dependent phosphorylation of PED/PEA-15 (Perfetti *et al.*, 2007), indicating that whether PKC induces or inhibits apoptosis depends on the particular subtype. Therefore, subtype identification is important for understanding the 20E signaling pathway.

Caspase-3, an apoptosis-related cysteine protease, is a key enzyme commonly found in the cell death pathways that respond to extracellular signals (Martin and Baehrecke, 2004). In vertebrates, caspase-3 activation releases caspase-activated DNase (CAD) from its inhibitor (inhibitor of caspase-activated DNase; ICAD), and the liberated CAD induces DNA fragmentation (Liu *et al.*, 1997; Enari *et al.*, 1998; Sakahira *et al.*, 1998). Since caspase-3 is highly conserved throughout the animal kingdom, caspase-3-like protease activity can be measured using a colorimetric substrate for human caspase-3 (Ilangovan *et al.*, 2003). An anti-human caspase-3 antibody exhibits strong cross-reactivity to the caspase-3-like protease of *Drosophila* (Yu *et al.*, 2002).

Using these tools, we showed that 20E-induced cell death includes activation of the caspase-3-like protease. A caspase-3 inhibitor inhibited DNA and nuclear fragmentation, showing that the caspase-3-like protease is involved in 20E signaling, as it is in cell death in other animals. Timed addition of the inhibitor showed that addition at 72 h, but not at 96 h, of 20E challenge prevented DNA and nuclear fragmentation. Caspase-3 activity measured using a colorimetric substrate also began to increase after 72 h and peaked at 96 h. Thus, the caspase-3-like protease may be activated between 72 and 96 h, in accordance with the timing of DNA fragmentation beginning at 96 h (Iga *et al.*, 2007).

The caspase-3-like protease involved in 20E-induced cell death appears somewhat different from human caspase-3. Western blot analysis of cultured *Bombyx* ASGs using anti-human caspase-3 antibody that recognizes the active fragment of caspase-3 revealed a single immunoreactive band. The intensity of this band increased dramatically between 72 and 96 h of 20E challenge, in accordance with the above-mentioned results. In human and *Drosophila*, proteolytic cleavage of

caspase-3 yields active fragments of 17 kDa (Han *et al.*, 1997) and 45 kDa, respectively; in *Bombyx*, the molecular weight of the fragment, estimated based on Western blotting, was 66 kDa (Kaneko and Sakurai, unpublished). These data indicate that the *Bombyx* caspase-3-like protease functions similarly to human caspase-3, but further understanding of the caspase-3-like protease awaits its identification and characterization.

Calmodulin/calmodulin-dependent protein kinase pathway

The 20E signaling pathway leading to nuclear (chromatin) condensation in *Bombyx* ASGs is distinct from the pathway leading to caspase-3-like protease-dependent DNA and nuclear fragmentation (Iga *et al.*, 2007). Similarly, in the *Drosophila* adult egg chamber, chromatin condensation occurs independently of any caspase-3-like protease activation in ovarian nurse or follicle cells and is controlled independently of DNA fragmentation (Nezis *et al.*, 2006). Induction of the apoptotic chromatin condensation has been suggested to result from Acinus activation by active caspase-3 (Sahara *et al.*, 1999). In *Bombyx* ASGs, the calmodulin antagonist W7, when added at 18 h of 20E challenge, inhibits induction of nuclear condensation as well as of DNA and nuclear fragmentation. Similarly, KN-93, an inhibitor of CaMKII, inhibited the condensation, indicating that calmodulin/CaMKII activation leads to nuclear condensation (Manaboon and Sakurai, unpublished).

Calmodulin/CaMKII is usually activated by Ca^{2+}, but in 20E-induced cell death, a Ca^{2+} ionophore mimicks 20E action by inducing DNA and nuclear fragmentation but not nuclear condensation. This result indicates that 20E signaling activates calmodulin/CaMKII independently of Ca^{2+} to regulate nuclear condensation. A calmodulin agonist and CaMKII inhibitor suppresses DNA and nuclear fragmentation to the same degree as that of the caspase-3 inhibitor, indicating that activation of the caspase-3-like protease in *Bombyx* may be dually regulated through PKC and CaMKII, although this issue is far from settled.

The calmodulin/CaMKII pathway is involved in cell death in mammals and insects. CaMKII activation is regulated by Ca^{2+}/calmodulin-dependent protein (CaM) binding or by autophosphorylation, which persists independently of Ca^{2+}. Although CaMKII autophosphorylation is initiated by binding of Ca^{2+}/CaM, Ca^{2+}/CaM-independent activity is substantial and is postulated to be important for synaptic and cellular plasticity in rat and *Drosophila* (Griffith, 2004; Elgersma *et al.*, 2004). In the central nervous system of *Drosophila*, the autophosphorylation ability of Ca^{2+}/CaMKII allows CaMKII to become Ca^{2+}-independent (Mehren and Griffith, 2004).

The protein phosphatase 1 and 2A (PP1/PP2) inhibitor calyculin A induces apoptosis, and the CaMKII inhibitor KN-93 blocks this effect of calyculin A. Calyculin A induces apoptosis by hyperphosphorylating CaMKII, suggesting that stringent limitation of CaMKII autophosphorylation restricts apoptosis (Fährmann *et al.*, 2007). Although the mechanism of caspase-3-like protease activation in ASGs is unknown, Ca^{2+}-independent, CaM-dependent activation of CaMKII may be involved.

Isoform-specific suppression of CaMKIIδc with the dominant negative-CaMKIIδc mutant, as well as nonselective CaMKII inhibition by KN-93, inhibits β1-adrenergic receptor-mediated stimulation of CaMKIIδc-mediated apoptosis in rat cardiomyocytes (Zhu *et al.*, 2007). In *Drosophila*, DmDopEcR is a homolog of the vertebrate γ-adrenergic receptors and is activated by 20E as well as dopamine. It has both a 20E-binding pocket and a dopamine-binding pocket. The receptor is coupled with $G_{\alpha s}$, and 20E activates the receptor as powerfully as dopamine (Srivastava *et al.*, 2005). These independent results indicate an involvement of mEcR in activation of CaMKII pathway in *Bombyx* ASG cell death.

CaMKII activation obviously mediates extracellular signal transduction in the cell death sequence, and Ca^{2+}/CaM-independent activation is brought about through autophosphorylation of CaMKII, although the initial phase of autophosphorylation requires mobilization of intracellular Ca^{2+}. In the death of *Bombyx* ASGs, a Ca^{2+} ionophore may not activate CaMKII, since it does not mimic 20E by inducing nuclear condensation, but inhibition of either calmodulin or CaMKII suppresses nuclear condensation (Iga *et al.*, 2007; Manaboon and Sakurai, unpublished), indicating that the mechanism involves Ca^{2+}-independent, calmodulin-dependent CaMKII activation.

Concluding remarks

Although our understanding of the nongenomic action of steroid hormones is limited, elucidation of its molecular mechanisms is important for understanding how steroid hormones exert their effects at quite different levels of biological events, including embryonic and post-embryonic development, reproduction, metabolism, homeostasis, and biological defense.

In eliciting its effects, 20E has interacting nongenomic and genomic actions. In *Choristoneura fumiferana* (spruce budworm), dequalinium-14;1,1′-decamethylenebis-4-aminoquinaldinium diiodide (DECA), an inhibitor of receptor of activated C kinase 1 (RACK1) binding to PKC, blocks 20E-induced expression of the transcription factor CHR3 by inhibiting translocation of EcR, probably by preventing its phosphorylation (Quan *et al.*, 2006). *CHR3* is an early-late gene and is a homologue of *BHR3* in *Bombyx* and *BgHR3* in *B. germanica*, the latter of which is involved in prothoracic gland cell death (Cruz *et al.*, 2006, 2007). Activation of the RACK1/PKC pathway is probably one of the nongenomic actions of 20E mediated by mEcR, and an example of the interaction between the genomic and nongenomic actions of 20E.

Nongenomic action of steroids may be involved, directly or indirectly, in regulating a variety of biological events. Elucidation of its underlying molecular mechanisms will contribute to the development not only of chemicals for insect

population control but also of therapies and drugs for syndromes that respond to steroid hormones. Until now, the molecular mechanisms underlying steroid action have been mostly understood as separate genomic and nongenomic mechanisms. We now recognize that this paradigm is inadequate. The elucidation of the interaction between the two signaling pathways may herald a new era for the study of steroid hormones.

References

Akai H. The structure and ultrastructure of the silk gland. Experientia 39: 443-449, 1983.

Applebaum SW, Gilbert LI. Stimulation of adenyl cyclase in pupal wing epidermis by β-ecdysone. Dev. Biol. 27: 165-175, 1972.

Baehrecke EH. Autophagic programmed cell death in *Drosophila*. Cell Death Differ. 10: 940-945, 2003.

Bender M, Imam FB, Talbot WS, Ganetzky B, Hogness DS. *Drosophila* ecdysone receptor mutations reveal functional differences among receptor isoforms. Cell 91: 777-788, 1997.

Boonyaratanakornkit V, Edwards DP. Receptor mechanisms mediating non-genomic actions of sex steroids. Semin. Reprod. Med. 5: 139-153, 2007.

Callus BA, Vaux DL. Caspase inhibitors: viral, cellular and chemical. Cell Death Differ. 14: 73-78, 2007.

Champlin DT, Truman JW. Ecdysteroid control of cell proliferation during optic lobe neurogenesis in the moth *Manduca sexta*. Development 125: 269-277, 1998.

Champlin DT, Truman JW. Ecdysteroid coordinates optic lobe neurogenesis via a nitric oxide signaling pathway. Development 127: 3543-3551, 2000.

Chinzei Y. Induction of histolysis by ecdysterone *in vitro*. Breakdown of anterior silk gland in silkworm, *Bombyx mori* (Lepidoptera, Bombycidae). Appl. Ent. Zool. 10: 136-138, 1975.

Cruz J, Mane-Padros D, Belles X, Martin D. Functions of the ecdysone receptor isoform-A in the hemimetabolous insect *Blattella germanica* revealed by systemic RNAi *in vivo*. Dev. Biol. 297: 158-171, 2006.

Cruz J, Martin D, Belles X. Redundant ecdysis regulatory functions of three nuclear receptor HR3 isoforms in the direct-developing insect *Blattella germanica*. Mech. Dev. 124: 180-189, 2007.

Daish TJ, Mills K, Kumar S. *Drosophila* caspase DRONC is required for specific developmental cell death pathways and stress-induced apoptosis. Dev. Cell 7: 909-915, 2004.

Davis MB, Carney GE, Robertson AE, Bender M. Phenotypic analysis of *EcR-A* mutants suggests that EcR isoforms have unique functions during *Drosophila* development. Dev. Biol. 282: 385-396, 2005.

Dorstyn L, Read SH, Quinni LM, Richardsoni H, Kumar S. DECAY, a novel *Drosophila* caspase related to mammalian caspase-3 and caspase-7. J. Biol. Chem. 274: 30778–30783, 1999.

Dubrovsky EB. Hormonal cross talk in insect development. Trends Endocrinol. Metab. 16: 6-11, 2005.

Elgersma Y, Sweatt JD, Giese KP. Mouse genetic approaches to investigating calcium/calmodulin-dependent protein kinase II function in plasticity and cognition. J. Neurosci. 24: 8410-8415, 2004.

Elmogy M, Iwami M, Sakurai S. Presence of membrane ecdysone receptor in the anterior silk gland of the silkworm *Bombyx mori*. Eur. J. Biochem. 271: 3171-3179, 2004.

Elmogy M, Iwami M, Sakurai S. Solubilization of the ecdysone binding protein from anterior silk gland cell membranes of the silkworm, *Bombyx mori*. Zool. Sci. 24: 971-977, 2007.

Elmogy M, Terashima J, Iga M, Iwami M, Sakurai S. A rapid increase in cAMP in response to 20-hydroxyecdysone in the anterior silk glands of the silkworm, *Bombyx mori*. Zool. Sci. 23: 715-719, 2006.

Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A, Nagata S. A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature 391: 43-50, 1998.

Fahrbach SE, Nambu JR, Schwartz LM. Programmed cell death in insect neuromuscular system during metamorphosis. In: Gilbert LI, Iatrou K, Gill SS (eds), Comprehensive molecular insect science, vol. 2. Elsevier, Oxford, pp 165-198, 2005.

Fährmann M, Honisch S, Kaufhold MA, Leitges M, Beil W. Stringent time-dependent transregulation of calcium calmodulin kinase II (CAMKII) is implicated in anti-apoptotic control. Biochim. Biophys. Acta [in press], 2007.

Griffith LC. Regulation of calcium/calmodulin-dependent protein kinase II activation by intramolecular and intermolecular interactions. J. Neurosci. 24: 8394-8398, 2004.

Han Z, Hendrickson EA, Bremner TA, Wyche JH. A sequential two-step mechanism for the production of the mature p17:p12 form of caspase-3 *in vitro*. J. Biol. Chem. 272: 13432-13436, 1997.

Henrich VC, Rybczynski R, Gilbert LI. Peptide hormones, steroid hormones, and puffs: Mechanisms and models in insect development. Vitam. Horm. 55: 73-125, 1999.

Herold MJ, McPherson KG, Reichardt HM. Glucocorticoids in T cell apoptosis and function. Cell. Mol. Life Sci. 63: 60–72, 2006.

Herr I, Gassler N, Friess H, Büchler MW. Regulation of differential pro- and anti-apoptotic signaling by glucocorticoids. Apoptosis 12: 271-291, 2007.

Hossain M, Shimizu S, Fujiwara H, Sakurai S, Iwami M. EcR expression in the prothoracicotropic hormone-producing neurosecretory cells of the *Bombyx mori* brain. FEBS J. 273: 3861-3868, 2006.

Iga M, Iwami M, Sakurai S. Nongenomic action of an insect steroid hormone in steroid-induced programmed cell death. Mol. Cell. Endocrinol. 263: 18-28, 2007.

Ilangovan R, Marshall WL, Hua Y, Zhou J. Inhibition of apoptosis by Z-VAD-fmk in SMN-depleted S2 cells. J. Biol. Chem. 278: 30993-30999, 2003.

Jiang C, Lamblin AF, Steller H, Thummel CS. A steroid-triggered transcription hierarchy controls salivary gland cell death during *Drosophila* metamorphosis. Mol. Cell 5: 445-455, 2000.

Kafatos FC, Tartakoff AM, Law JH. Cocoonase. I. Preliminary characterization of a proteolytic enzyme from silk moth. J. Biol. Chem. 242: 1477-1487, 1967.

Kakei M, Iwami M, Sakurai S. Death commitment in the anterior silk gland of the silkworm, *Bombyx mori*. J. Insect Physiol. 51: 17-25, 2005.

Kamimura M, Tomita S, Kiuchi M, Fujiwara H. Tissue-specific and stage-specific expression of two silkworm ecdysone receptor isoforms. Ecdysteroid-dependent transcription in cultured anterior silk glands. Eur. J. Biochem. 248: 786-793, 1997.

Koyama T, Iwami M, Sakurai S. Ecdysteroid control of cell cycle and cellular commitment in insect wing imaginal discs. Mol. Cell. Endocrinol. 213: 155-166, 2003.

Kubo K, Matsuda Y, Kase H, Yamada K. Inhibition of calmodulion-dependent cyclic nucleotide phosphodiesterase by flunarizine, a calcium-entry blocker. Biochem. Biophys. Res. Commun. 124: 315-321, 1984.

Lam G, Hall BL, Bender M, Thummel CS. *DHR3* is required for the prepupal-pupal transition and differentiation of adult structures during *Drosophila* metamorphosis. Dev. Biol. 212: 204-216, 1999.

Lee CY, Baehrecke EH. Steroid regulation of autophagic programmed cell death during development. Development 28: 1443-1455, 2001.

Lee CY, Clough EA, Yellon P, Teslovich TM, Stephan DA, Baehrecke EH. Genome-wide analyses of steroid- and radiation-triggered programmed cell death in *Drosophila*. Curr. Biol. 13: 350-357, 2003.

Leulier F, Ribeiro PS, Palmer E, Tenev T, Takahashi K, Robertson D, *et al.* Systematic *in vivo* RNAi analysis of putative components of the *Drosophila* cell death machinery. Cell Death Differ. 13: 1663-1674, 2006.

Lieberherr M, Grosse B. Androgens increase intracellular calcium concentration and inisitol 1,4,5-phosphate and diacylglycerol formation via a pertussis toxin-sensitive G-protein. J. Biol. Chem. 269: 7217-7223, 1994.

Liu X, Zou H, Slaughter C, Wang X. DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. Cell 89: 175-184, 1997.

Lösel R, Falenstein E, Feuring M, Schltz A, Tillmann HC, Rossel-Haseroth K, *et al.* Nongenomic steroid action: controversies, questions, and answers. Physiol. Rev. 83: 965-1016, 2003.

Lösel R, Wehling M. Nongenomic actions of steroid hormones. Nat. Rev. Mol. Cell Biol. 4: 46-56, 2003.

Löwenberg M, Verhaar AP, van den Brink GR, Hommes DW. Glucocorticoid signaling: a nongenomic mechanism for T-cell immunosuppression. Trends Mol. Med. 13: 157-163, 2007.

Mane-Padros D, Cruz J, Vilaplana L, Pascual N, Belles X, Martin D. The nuclear hormone receptor BgE75 links molting and developmental progression in the direct-developing insect *Blattella germanica*. Dev. Biol. [in press], 2008.

Martin DN, Baehrecke EH. Caspases function in autophagic programmed cell death in *Drosophila*. Development 131: 275-284, 2004.

Maruko T, Nakahara T, Sakamoto K, Saito M, Sugimoto N, Takuwa Y, *et al.* Involvement of the subunits of G proteins in the cAMP response induced by stimulation of the histamine H1 receptor. Naunyn-Schmiedegerg's Arch. Pharmacol. 372: 153-159, 2005.

Mehren JE, Griffith LC. Calcium-independent calcium/calmodulin-dependent protein kinase II in the adult *Drosophila* CNS enhances the training of pheromonal cues. J. Neurosci. 24: 10584-10593, 2004.

Minakuchi C, Nakagawa Y, Kiuchi M, Tomita S, Kamimura M. Molecular cloning, expression analysis and functional confirmation of two ecdysone receptor isoforms from the rice stem borer *Chilo suppressalis*. Insect Biochem. Mol. Biol. 32: 999-1008, 2002.

Nezis IP, Stravopodis DJ, Margaritis LH, Papassideri IS. Chromatin condensation of ovarian nurse and follicle cells is regulated independently from DNA fragmentation during *Drosophila* late oogenesis. Differentiation 74: 293-304, 2006.

Obara Y, Miyatani M, Ishiguro Y, Hirota K, Koyama T, Izumi S, *et al.* Pupal commitment and its hormonal control in wing imaginal discs. J. Insect Physiol. 48: 933-944, 2002.

Pace MC, Thomas P. Activation of a pertussis toxin-sensitive, inhibitory G-protein is necessary for steroid-mediated oocyte maturation in spotted seatrout. Dev. Biol. 285: 70-79, 2005.

Palanker L, Necakov AS, Sampson HM, Ni R, Hu C, Thummel CS, *et al.* Dynamic regulation of *Drosophila* nuclear receptor activity *in vivo*. Development 133: 3549-3562, 2006.

Perfetti A, Oriente F, Iovino S, Alberobello AT, Barbagallo AP, Esposito I, *et al.* Phorbol esters induce intracellular accumulation of the anti-apoptotic protein PED/PEA-15 by preventing ubiquitinylation and proteasomal degradation. J. Biol. Chem. 282: 8648-8657, 2007.

Qiu J, Bosch MA, Jamali K, Xue C, Kelly MJ, Rønnekleiv OK. Estrogen upregulates T-type calcium channels in the hypothalamus and pituitary. J. Neurosci. 26: 11072-11082, 2006.

Quan GX, Krell PJ, Arif BM, Feng Q. Receptor of activated C kinase 1 (RACK1) is necessary for the 20-hydroxyecdysone-induced expression of the transcription factor CHR3 in the spruce budworm *Choristoneura fumiferana*. Insect Mol. Biol. 15: 79-87, 2006.

Revankar CM, Cimino DF, Sklar LA, Arterburn JB, Prossnitz ER. A transmembrane intracellular estrogen receptor mediates rapid cell signaling. Science 307: 1625-1630, 2005.

Riddiford LM. Cellular and molecular actions of juvenile hormone I. General considerations and premetamorphic actions. Adv. Insect Physiol. 24: 213-274, 1994.

Riddiford LM. Juvenile hormone: the status of its "status quo" action. Arch. Insect Biochem. Physiol. 32: 271-286, 1996.
Rønnekleiv OK, Kelly MJ. Diversity of ovarian steroid signaling in the hypothalamus. Front. Neuroendocrinol. 26: 65-84, 2005.
Sahara S, Aoto M, Eguchi Y, Imamoto N, Yoneda Y, Tsujimoto Y. Acinus is a caspase-3-activated protein required for apoptotic chromatin condensation. Nature 401: 168-173, 1999.
Sakahira H, Enari M, Nagata S. Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. Nature 391: 96-99, 1998.
Sakurai S, Kaya M, Satake S. Hemolymph ecdysteroid titer and ecdysteroid-dependent developmental events in the last-larval stadium of the silkworm, *Bombyx mori*: Role of low ecdysteroid titer in larval-pupal metamorphosis and a reappraisal of the head critical period. J. Insect Physiol. 44: 867-881, 1998.
Schlattner U, Vafopoulou X, Steel CG, Hormann RE, Lezzi M. Non-genomic ecdysone effects and the invertebrate nuclear steroid hormone receptor EcR--new role for an "old" receptor? Mol. Cell Endocrinol. 247: 64-72, 2006.
Sekimoto T, Iwami M, Sakurai S. Coordinate responses of transcription factors to ecdysone during programmed cell death in the anterior silk gland of the silkworm, *Bombyx mori*. Insect Mol. Biol. 15: 281-292, 2006.
Sekimoto T, Iwami M, Sakurai S. 20-Hydroxyecdysone regulation of two isoforms of the Ets transcription factor *E74* gene in programmed cell death in the silkworm anterior silk gland. Insect Mol. Biol. 16: 581-590, 2007.
Sergeev IN. Calcium signaling in cancer and vitamin D. J. Steroid Biochem. Mol. Biol. 97: 145-151, 2005.
Simoncini T, Fornari L, Mannella P, Varone G, Garuso A, Liao JK, *et al.* Novel non-transcriptional mechanisms for estrogen receptor signaling in the cardiovascular system. Interaction of estrogen receptor alpha with phosphatidylinositol 3-OH kinase. Steroids 67: 935-939, 2002.
Simoncini T, Mannella P, Fornari L, Caruso A, Varone G, Genazzani AR. Genomic and non-genomic effects of estrogens on endothelial cells. Steroids 69: 537-542, 2004.
Srivastava DP, Yu EJ, Kennedy K, Chatwin H, Reale V, Hamon M, *et al.* Rapid, nongenomic responses to ecdysteroids and catecholamines mediated by a novel *Drosophila* G-protein-coupled receptor. J. Neurosci. 25: 6145-6155, 2005.
Suyama E, Matsunaga T, Shirai H, Fujiwara H. Pupal wing morphogenesis controlled by region-specific expression of ecdysone receptor (EcR) isoforms. Zool. Sci. 20: 1609, 2003.
Talbot WS, Swyryd EA, Hogness DS. *Drosophila* tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms. Cell 73: 1323-1337, 1993.
Tasker JG, Di S, Malcher-Lopes R. Rapid glucocorticoid signaling via membrane-associated receptors. Endocrinol. 147: 5549-5556, 2006.
Terashima J, Yasuhara N, Iwami M, Sakurai S. Programmed cell death triggered by insect steroid hormone, 20-hydroxyecdysone, in the anterior silk gland of the silkworm, *Bombyx mori*. Dev. Genes Evol. 11: 545-558, 2000.
Thomas P, Pang Y, Filardo EJ, Dong J. Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. Endocrinol. 146: 624-632, 2005.
Thomas P, Pang Y, Groenen P, Kelder J, Dong J, Zhu Y, *et al*. Steroid and G protein binding characteristics of the seatrout and human progestin membrane receptor alpha subtypes and their evolutionary origins. Endocrinol. 148: 705-718, 2007.
Tomaschko KH. Nongenomic effects of ecdysteroids. Arch. Insect Biochem. Physiol. 41: 89-98, 1999.
Wehling M. Specific, nongenomic actions of steroid hormones. Ann. Rev. Physiol. 59: 365-393, 1997.
Winoto A, Littman DR. Nuclear hormone receptors in T lymphocytes. Cell 109 (Suppl): S57-66, 2002.
Woo M, Hakem R, Soengas MS, Duncan GS, Shahinian A, Kagi D, *et al*. Essential contribution of caspase 3/CPP32 to apoptosis and its associated nuclear changes. Genes Dev. 12: 806-819, 1998.
Yao TP, Segraves WA, Oro AE, MaKeown M, Evans RM. *Drosophila* ultraspiracle modulates ecdysone receptor function via heterodimer formation. Cell 71: 63-72, 1992.
Yin VP, Thummel CS. Mechanisms of steroid-triggered programmed cell death in *Drosophila*. Semin. Cell Dev. Biol. 16: 237-243, 2005.
Yin VP, Thummel CS. Down-regulation of inhibitor of apoptosis levels provides competence for steroid-triggered cell death. J. Cell Biol. 178: 85-92, 2007.
Yu SY, Yoo SJ, Yang L, Zapata C, Srinivasan A, Hay BA, *et al.* A pathway of signals regulating effector and initiator caspases in the developing *Drosophila* eye. Development 129: 3269-3278, 2002.
Zhu W, Woo AY, Yang D, Cheng H, Crow MT, Xiao RP. Activation of CaMKIIδc is a common intermediate of diverse death stimuli-induced heart muscle cell apoptosis. J. Biol. Chem. 282: 10833-10839, 2007.
Zhu Y, Rice CD, Pang Y, Pace M, Thomas P. Cloning, expression, and characterization of a membrane progestin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes. Proc. Natl. Acad. Sci. USA 100: 2231-2236, 2003.
Zivadinovic D, Gametchu B, Watson CS. Membrane estrogen receptor-α levels in MCF-7 breast cancer cells predict cAMP and proliferation responses. Breast Cancer Res. 7: R101-112, 2005.