

Proteomic identification of differentially expressed and
phosphorylated proteins in 20-hydroxyecdysone (20E) signal
transduction pathway in salivary gland of *Drosophila melanogaster*

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Proteomics identification of differentially expressed and phosphorylated proteins in 20-hydroxyecdysone (20E) signal transduction pathway in salivary gland of *Drosophila melanogaster*

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ABSTRACT

Protein kinase C (PKC) plays important role in insect molting hormone signal transduction, however, little is known about the exact role of PKC in this process. In my research, PKC-regulated phosphorylation in molting hormone signal transduction is investigated in the salivary gland of *Drosophila melanogaster* (fruit fly). Our experiments demonstrate that PKC-regulated phosphorylation is responsible for the intracellular localization of the subunits of molting hormone receptor complex, which is possibly due to the forming of a more complicated receptor complex with chaperons. We also confirmed PKC-regulated phosphorylation is required in molting hormone induced protein expression and identified 14 proteins induced by molting hormone but inhibited by a PKC inhibitor. Using 2D Western blot and phospho-(Ser) PKC substrate antibody, we were able to identify four phosphorylated PKC substrates in 20E signal transduction process, which may function in 20E-induced gene transcription/translation process or in ecdysteroid transporting. In addition, PKC isoforms in the salivary gland were also investigated by RNA interference (RNAi). For the first time, we showed the successful application of RNAi technology by soaking the salivary glands of *D. melanogaster* with dsRNAs.

CHAPTER 1

Introduction

Insects, the biggest class of arthropods, share a feature with all other arthropods—molting. Molting is an intrinsic requirement of animals with exoskeletons, which allows them to expand their bodies under controlled and protected conditions. The exoskeleton of insects is secreted by the epidermis and is a composite of several kinds of bio-functional molecules, primarily a polymeric structure of protein and chitin chains with trace amounts of phenolics, lipids and minerals. Although the exoskeleton provides comprehensive protection to insects from water loss, external physical injury and pathogens, it has a vital defect: the exoskeleton is not expandable and constrains the body size of insects when they grow to a certain size. Therefore, molting, a sophisticated and effective physiological process, occurs at certain stages of insects' lives, allowing insects to shed the old cuticles and replace them with new larger ones periodically. This process is repeated several times during the post-embryonic stages of insects until they become adults.

By molting, insects not only gain larger bodies, but sometimes transform their external structure, the latter is called metamorphosis. The metamorphosis of insects, depending on different types of morphological change, can be divided into two sets: complete metamorphosis and incomplete metamorphosis. Insects with complete metamorphosis pass through four different development stages: egg or embryo, larva, pupa and adult. The larvae bear no resemblance to their adult parents in complete metamorphosis. Over 85% of insects go through complete metamorphosis. Beetles (Order Coleoptera), butterflies and moths (Order Lepidoptera), flies (Order Diptera) and wasps & bees (Order Hymenoptera) are examples of complete metamorphosis. Insects that undergo incomplete metamorphosis lack a pupa stage. And the immature insects are usually called nymphs instead of larvae, which in most cases resemble their parents in their appearances. About 15% of insects go through incomplete metamorphosis. Incomplete metamorphosis consists of three life stages: egg, nymph and adult. Notable examples are grasshoppers, crickets, cockroaches (Order Orthoptera), true bugs (Order Hemiptera), cicadas, hoppers, aphids (Order Homoptera), and termites (Order Isoptera)(Wheeler et al. 2001).

1.1 Molting process

Molting includes two steps — apolysis and ecdysis. Apolysis, the first step of molting, refers to the detachment of the epidermis from the overlying cuticle. In this process, epidermis secretes a molting fluid into the space between the epidermis and old cuticle and then proceeds to secrete the layers of new cuticle. After the formation of new epicuticle, proenzymes in the molting fluid are activated and degrade the old cuticle from inside out except the highly cross-linked outer exocuticle. The molting fluid is then reabsorbed when the digestion is completed. Once apolysis is accomplished, the molting process proceeds to the 2nd step—ecdysis, which is the process of an insect shedding the old cuticle. By crawling movements, the pharate insects push forward in the old cuticle, which splits down the back allowing the animal to emerge. Often, this initial crack is caused by an increase in blood pressure within the body (in combination with movement), forcing an expansion across its exoskeleton, and leading to an eventual crack that allows insects to extricate themselves. After insects shed their old cuticles, they then inflate their new cuticles to their final forms. The proteins in this expanded cuticle are then cross-linked by a quinone-based tanning and/or

β sclerotization, and the new size and shape of the insects are then fixed until their next molting (Nation 2007).

1.2 Hormones involved in molting

Molting has been known as a hormone-controlled physiological process since 1917 (Kopeć 1917; Kopeć 1922). Wigglesworth started his investigations on the control of molting and metamorphosis in the blood sucking bug, *Rhodnius prolixus*. His research tested the hypothesis of Kopeć that hormones produced by the brain are critical to insect molting (Wigglesworth 1934). Later, ligation experiments in Lepidoptera verified the results of Wigglesworth's experiments. In Kuhn and Piepho's experiment, ligation of the head part of caterpillars prevented the molting, while the implantation of the brain into the abdomen could restore molting (Kühn 1936). Research on the mechanism of hormone-controlled molting started with studies on the ecdysis of giant silk moths 40 years ago. Truman and Riddiford designed brain extirpation and implantation experiments within and between two species of giant silk moths, *Hyalophora cecropia* and *Antheraea pernyi*, which showed that the brain controlled the timing of the

behavior and exerted this control even if transplanted to the abdomen (Truman and Riddiford 1970). The research then shifted to the tobacco hornworm moth, *Manduca sexta*. Researches in *M. sexta* provided a growing list of peptide hormones that orchestrate the ecdysis sequence. In recent years, *Drosophila melanogaster* (fruit fly) has been used as a major subject to determine the function of specific peptides and to identify new components in this regulatory pathway.

1.2.1 Molting hormone-20 hydroxyecdysone

Ecdysone was first isolated by Butenandt and Karlson (1954) from 500 kilograms silkworm pupae. This became the first isolated invertebrate hormone in history. Soon, more ecdysone-like substances were discovered not only in invertebrates, but in plants and vertebrates; these ecdysone-like chemicals then were named as a family, ecdysteroids (**Fig.1**). Ecdysteroids are present in animals (zooecdysteroids), plants (phytoecdysteroids) and fungi (mycoecdysteroids).

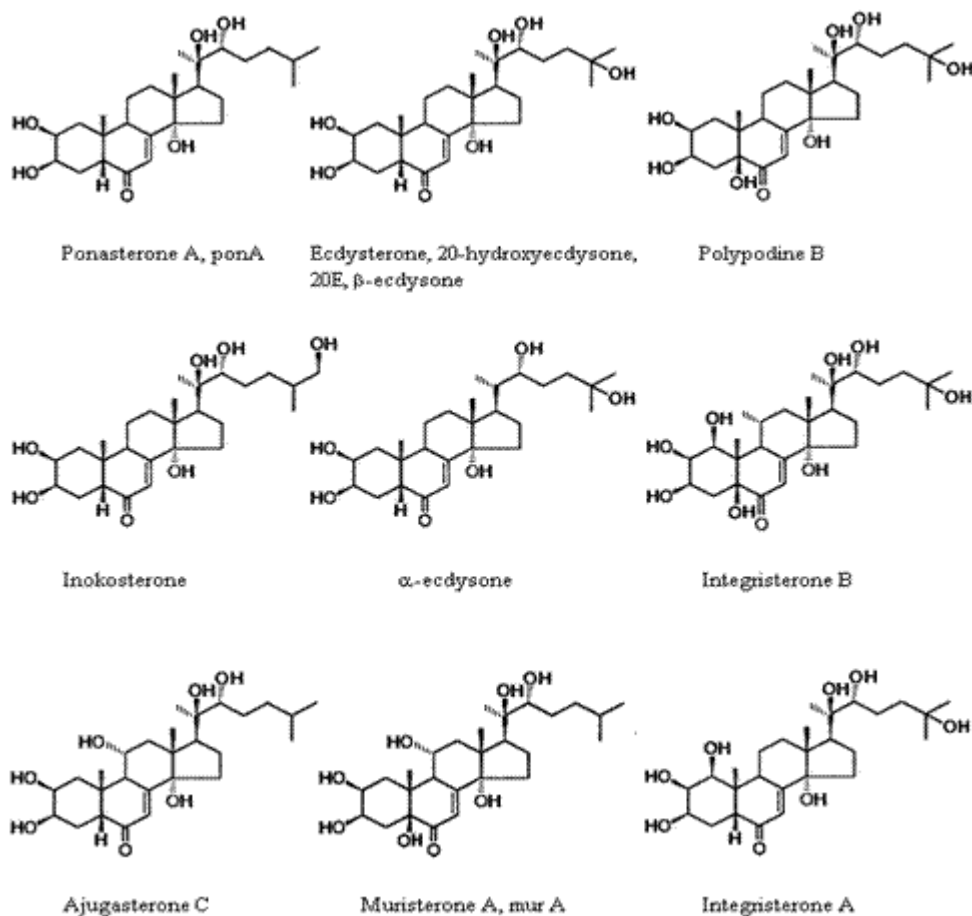


Fig. 1. Representatives of ecdysteroids
 (From http://leuzea.ru/leuzea_ecdysteroids.htm)

At the beginning, ecdysone was identified as the molting hormone in insects; however, the later research showed, 20-hydroxyecdysone (20E), not ecdysone, is the functional molting hormone. 20E alone can induce the molting of insects in the larval stage. 20E is also called β -ecdysone in the older literature, because the hydroxyl group at carbon-20 is in the β -configuration. Ecdysone is the precursor

of 20E. After secretion by the prothoracic gland, ecdysone is transported in hemolymph to tissues where it is converted to 20E via 20-hydroxymonooxygenase which adds a hydroxyl group to ecdysone. Among the hundreds of ecdysteroids identified in insects and plants, 20E is the most active and widely used ecdysteroid in insect molting. In the following content, we use '20E' to indicate 'ecdysteroid'. 20E titer in insects is essential to trigger the molting process. **Fig. 2** depicts ecdysteroid (20E) titer in *D. melanogaster* from embryo to adult. Molting processes happen after each peak of 20E in the development of *D. melanogaster*, from embryo to larva, larva to larva, larva to pupa, and pupa to adult.

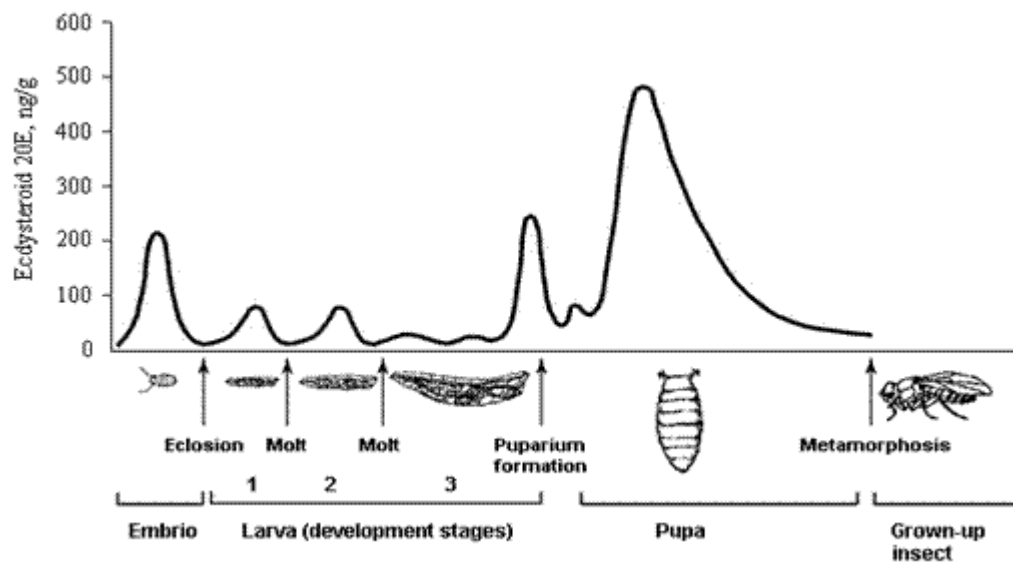


Fig. 2. 20E titer in the *D. melanogaster* development (Kozlova and Thummel 2000).

Though 20E is essential for insect molting, insects themselves do not synthesize 20E from simple precursors, such as acetate and mevalonate. Insects produce 20E by ingesting one of a variety of steroids. In the case of plant-eating insects (phytophagous), the steroids are mainly sitosterol and campesterol, which are dealkylated by insects to produce cholesterol and then concentrated in some insect specific tissues (e.g. ring gland). On the other hand, carnivorous insects gain their cholesterol directly from other animals (Gilbert et al. 2002).

1.2.2 Juvenile hormone (JH)

Juvenile hormone (JH) was first discovered in the blood sucking bug, *Rhodnius prolix*, by Wigglesworth (1934), and was thought to be a humoral factor that could inhibit metamorphosis from the last instar nymph to adult. Several insect JHs have been identified, which form a group of acyclic sesquiterpenoids, including JH 0, JH I, JH II, JH III (**Fig. 3**). JH acts in many aspects of insect development, such as body development, reproduction, diapause and polyphenism. The name of JH came from one of its main functions: keeping insects in their juvenile stage. JH does not trigger the molting process directly;

however, by interacting with 20E, JH orchestrates the development of insects, especially the timing and method of molting (metamorphosis).

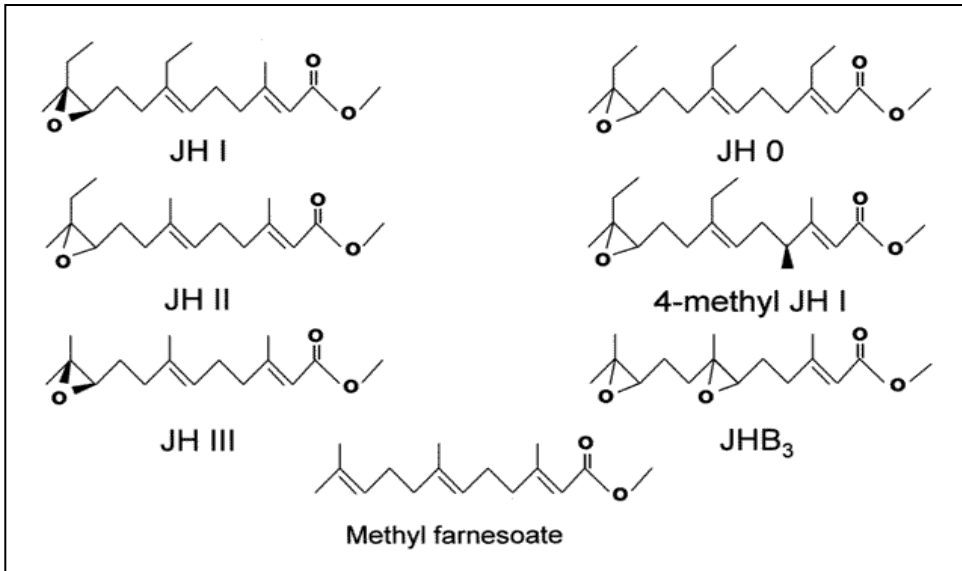


Fig. 3. Structures of the common insect juvenile hormones and cognates (From webpage of Walter Goodman, <http://entomology.wisc.edu/~goodman/wgresrch.html>).

The titer of JH in the hemolymph of the developing insect determines the development stage of insects. Generally, high JH titer is prone to keep insects in larval forms. JH level gradually decreases during the development of insects, allowing insects to proceed to successive instars with each molting and the metamorphosis. Although JH have been discovered and studied for several decades, the JH receptor is still unknown.

1.2.3 Cross-talk of 20E and JH

Molting is a very complicated physiological process, and requires the coordination of multiple hormones, including 20E, JH, prothoracicotropic hormone (PTTH), eclosion hormone (EH), ecdysis triggering hormone (ETH), bursicon, etc. Among these hormones, 20E and JH are the two major hormones that orchestrate the molting process. A general scheme of this hormone controlled molting process is described below (Fig. 4). Prothoracicotropic hormone (PTTH) triggers the synthesis and release of 20E precursor, ecdysone, from the prothoracic gland, and then ecdysone is converted to the active form, 20E, by a 20-monooxidase located in the mitochondria of larval fat body and in other tissues (Mitchell and Smith 1988). An increase of 20E initiates the molting process. The nature of molting is determined by the titer of JH, which decreases with the growth of insects. At the beginning of an insect life cycle, a larva encounters its first 20E peak with a high JH titer and replaces the 1st instar larval cuticle with 2nd instar larval cuticle. With the development of the larva, JH titer declines, but still enough to control the molting from one larval stage to the next larval stage. When the larva grows to a certain size, JH reaches its low point, and the peak(s) of 20E initiates the molting from larva to pupa with the larval cuticle

replaced by pupal cuticle. After head eversion, the pupal stage is completed. The onset of the last molting, namely metamorphosis, is triggered by a major peak of 20E and the absence of JH (**Fig. 4**).

The mechanism of crosstalk between 20E and JH at the molecular level is not very clear. Several theories have been proposed to explain this mechanism, but no convincing explanation with solid evidence has yet been proposed. Two popular theories, based on the two hypothesized JH receptors, have been suggested. One theory is based on the assumption that the ultraspiracle protein (USP), a heterodimer of the ecdysone receptor (EcR), is not only a component of the ecdysone receptor complex, but is also the receptor of JH. In this theory,

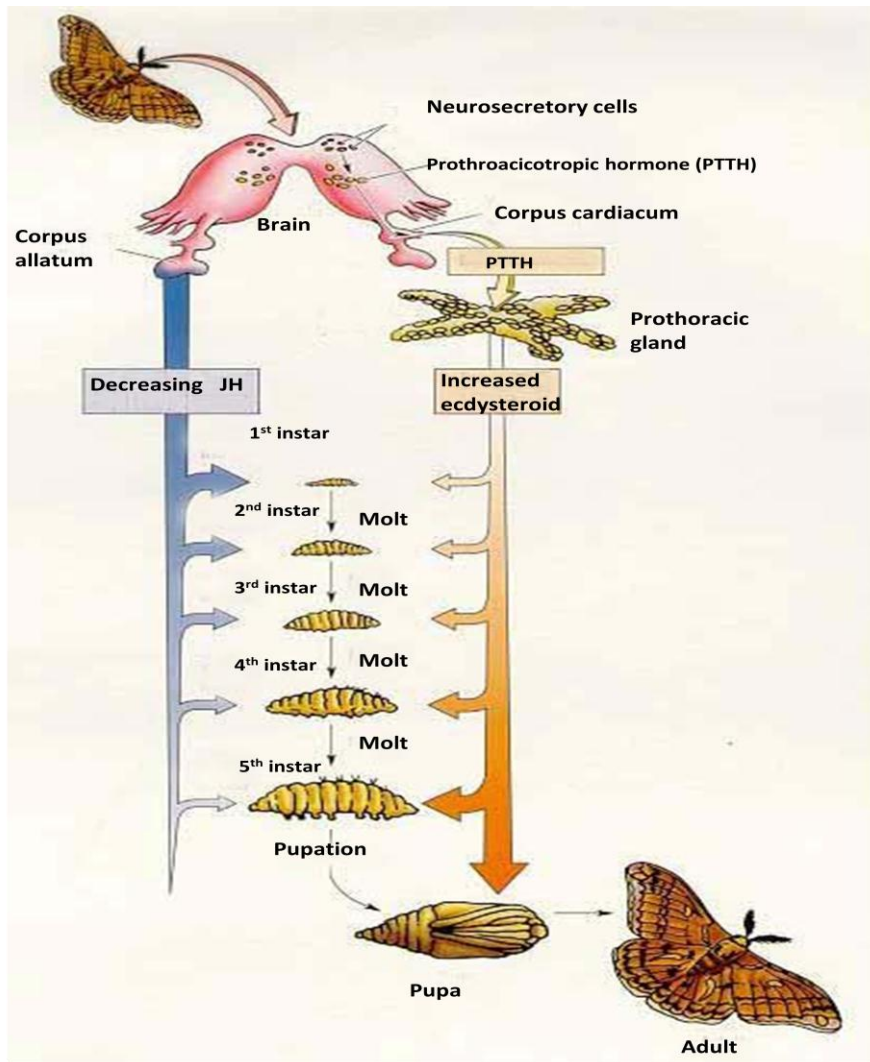


Fig. 4. Interaction of ecdysteroid and JH in insect development (Wigglesworth, 1984).

Researchers hypothesize that JH is able to bind to the ligand-binding domain of USP. In the presence of JH on USP, binding of 20E on EcR can only activate larval molting; whereas in the absence of JH, 20E alone stimulates the process of metamorphosis (Fang et al. 2005) (**Fig. 5A**). The problem with this theory is that

USP has much weaker affinity for JH than expected for classical nuclear receptors. The second theory assumes that methoprene-tolerant (MET) is the receptor of JH. When JH is present, MET binds JH and remains as a monomer and regulates larval genes. 20E stimulation will only initiate a molting to next larval stage. When JH is absent, Met either homodimerizes or heterodimerizes with germ cells-expressed (GCE) and metamorphosis will proceed in response to 20E (Miura et al. 2005; Godlewski et al. 2006) (**Fig. 5B**). The above two theories suggest that JH receptor is the key to elucidate the true mechanism of crosstalk between JH and 20E.

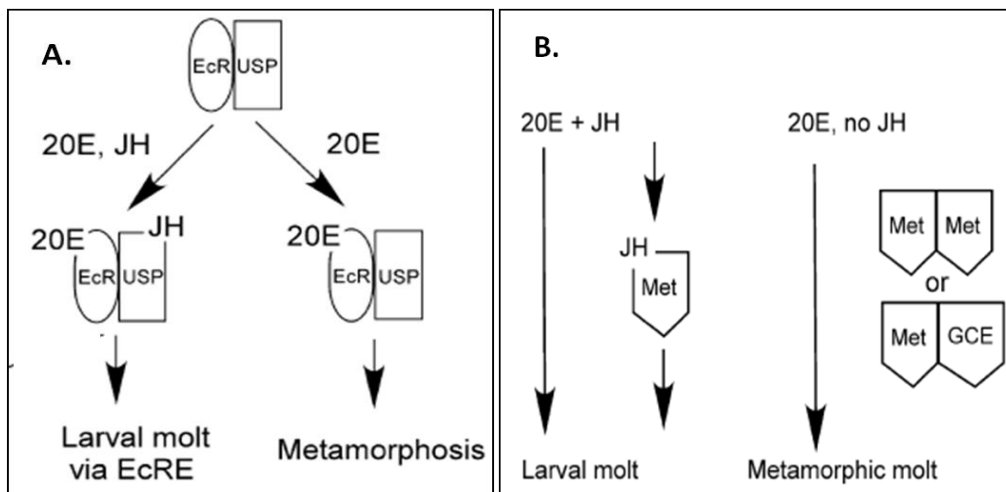


Fig. 5. Two proposed models of crosstalk of 20E and JH. **A.** Proposed model of USP as JH receptor in crosstalk between 20E and JH. EcR, ecdysone receptor; EcRE, ecdysone response element to which EcR-USP binds; USP, Ultraspiracle; **B.** Model for Methoprene-tolerant (Met) as the JH receptor in crosstalk between 20E and JH. Met, Methoprene-tolerant; GCE, germ cells-expressed. Modified from (Riddiford 2008).

1.3 The mode action of 20E

Like most steroid hormones, 20E triggers molting through a receptor complex.

However, unlike mammalian steroid hormone receptors, which bind to their response elements as a homodimer, the insect ecdysteroid receptor complex is a heterodimer, consisting of ecdysteroid receptor (EcR) and ultraspiracle protein (USP), an orthologue of the vertebrate retinoid X receptor (RXR). This is similar to thyroid hormone receptors (TR) and retinoic acid receptors (RAR) in mammals, which form heterodimers with their partner, RXR. Both EcR and USP belong to the nuclear receptors super family (Riddiford et al. 2000). The EcR-USP complex binds ecdysteroid and then activates the transcription of ecdysteroid-responsive genes by binding to the ecdysteroid response element (EcRE) on DNA (Fig. 6).

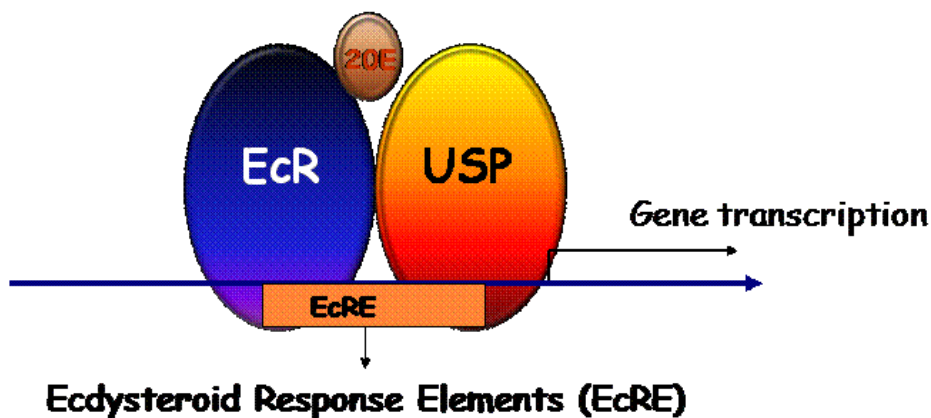


Fig. 6. 20E-induced gene expression. 20E activated gene transcription through the binding of ecdysteroid receptor complex (EcR-USP) on ecdysteroid response elements (EcRE) on DNA.

1.3.1 Ecdysteroid receptor (EcR)

The *EcR* gene, encoding the ecdysteroid receptor (EcR), was cloned in *D.*

melanogaster in 1991 (Koelle et al. 1991), which provided a major advance to the study of insect metamorphosis at the molecular level. EcR, then was shown to be a member of the nuclear receptor superfamily (Mangelsdorf and Evans 1995; Mangelsdorf et al. 1995). When comparing the amino acid sequences of the DNA binding domain to different nuclear receptors, EcR shows the most similarity with a subfamily of steroid receptors in vertebrates, which includes TR, RAR, vitamin D receptor and other hormone receptors. All of the most highly conserved amino acids in the DNA binding domain of steroid receptors in vertebrates are present in EcR, including the nine cysteine residues. Eight out of the nine cysteine residues are involved in forming two zinc fingers, which are the key structures for the receptor's DNA binding function. The whole amino acid sequence of EcR shows the typical modular structure of a nuclear receptor: N-terminal A/B domain, DNA-binding C domain, the hinge D region, the ligand-

binding E domain, and the C-terminal F domain. The *EcR* gene encodes three protein isoforms in *D. melanogaster*: EcR-A, EcR-B1 and EcR-B2 (**Fig. 7**). These isoforms have a common carboxy-terminal region, including DNA-binding and ligand-binding domains, but different amino terminals.

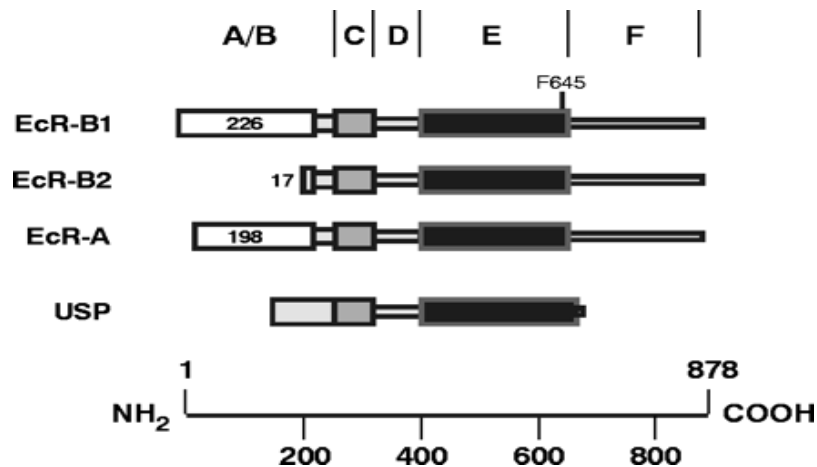


Fig. 7. Domain structures of the ecdysone receptor components. The three isoforms of *Drosophila* EcR and the single *Drosophila* USP are shown, with the standard nuclear receptor regions indicated. Region C is the DNA-binding domain (DBD); region E, the ligand-binding domain (LBD). The three EcR isoforms are identical in sequence except in the A/B regions that are unrelated. The number of isoform-specific residues is shown for each EcR. (Cherbas et al. 2003)

Each EcR isoform can heterodimerize with USP to form an ecdysteroid receptor complex (Koelle, 1992). In *D. melanogaster*, Talbot et al. showed that EcR-A is predominantly expressed in adult progenitor cells that proliferate and differentiate during metamorphosis, while the two EcR-B isoforms are

predominantly expressed in the larval cells fated to die. The researchers also determined the temporal expression profiles of EcR isoforms. The results indicated that different isoforms predominate at different developmental stages marked by a pulse of ecdysone (Talbot et al. 1993). This observation led to the hypothesis that different EcR isoforms control at least part of the tissue and development stage specificity of ecdysone responses. This hypothesis was then confirmed in many other studies (Robinow et al. 1993; Truman et al. 1994; Kim et al. 1999). Research on EcR isoform mutants also supported this hypothesis. It has been shown that the leg imaginal discs elongated in *EcR-B* mutants, while the larval tissues failed to die (Bender et al. 1997; Schubiger et al. 1998). In *EcR-A* mutants, the salivary gland was unable to degenerate and the mutants died during middle or late pupation stages. (Davis et al. 2005). All evidence suggests that insects generate stage/tissue-specific physiological responses to ecdysteroid at specific development stages and specific tissues in their life cycle through different EcR isoforms.

1.3.2 Ultra spiracle protein (USP)

USP was first characterized in *D. melanogaster* as a mammalian retinoid X receptor homologue, and was thought to carry out similar regulatory functions as its vertebrate counterparts (Henrich et al. 1990; Oro et al. 1990). Later, with the identification of the first molting hormone receptor, EcR, the researchers found that USP is the heterodimeric partner of EcR. USP and EcR form a receptor complex which enables the transactivation of ecdysteroid response genes (Yao et al. 1992; Thomas et al. 1993; Yao et al. 1993). The cell extracts containing only *Drosophila* EcR showed a 90-fold lower ligand binding affinity compared to heterodimerization with USP. So, the biological significance of hormone binding of unpartnered EcR was often considered to be negligible.

EcR is not the only heterodimeric partner of USP. In fruit flies, DHR38, another nuclear receptor, has been demonstrated to have the ability to compete with EcR *in vitro* to dimerize with USP and consequently disrupt EcR-USP binding to an EcRE. Moreover, transfection experiments in Schneider cells showed that DHR38 could affect ecdysone-dependent transcription (Sutherland et al. 1995). Similarly, Seven-up (Svp), the *Drosophila* homologue of the chicken ovalbumin upstream transcription factor, also modulates ultraspiracle-based hormonal

signaling both *in vitro* and *in vivo*, either through DNA binding competition or protein-protein interactions (Zelhof et al. 1995). Another role USP may play is as JH receptor, as mentioned in **1.2.4**. Thus, USP has been implicated to be a cross point of both 20E and JH signal transduction pathways.

Similarly to EcRs, multiple USP isoforms have been identified in different insects. The first report of multiple USP forms came from the mosquito, *A. aegypti*, in which AaUSPa and AaUSPb were cloned and characterized (Kapitskaya et al. 1996). Then, two USP isoforms were found in *M. sexta* and *Chironomus. tentans*, USP1 and USP2 (Vogtli et al. 1999), but only one form has been found in *D. melanogaster*. To this date, we still know little about the link between functions and structures of USP isoforms. In summary, USP plays a central role as an ubiquitous heterodimerization partner of many nuclear receptors in insects. Besides ecdysteroid signal transduction, it is highly possible to be involved in many other physiological processes.

1.3.3 The EcR-USP receptor complex

Although EcR can bind the ligand (i.e. 20E) without heterodimerization with USP (Christopherson et al. 1992; Grebe et al. 2004), the ligand binding can be greatly enhanced by USP. The ligand binding affinity of EcR is often measured by the use of radiolabeled ponasterone A (PonA), and the specific binding of PonA to EcR was drastically enhanced by the addition of USP (Minakuchi et al. 2003; Ogura et al. 2005). In addition, the ligand binding was found to increase the EcR-USP heterodimer's stability and its affinity to 20E-response elements (Yao et al. 1993). Thus, the EcR-USP heterodimer is still considered to be the main functional ecdysteroid receptor.

1.3.4 Intracellular localization of EcR and USP

Nuclear receptors exert their transactivation function in nuclei; however, the receptors themselves are synthesized in cytoplasm. Thus, nuclear transport is important to the receptors. Some nuclear receptors are transported to the nucleus immediately after synthesis, while others may need stimulation. Nuclear receptors can be categorized in three groups according to their distributions: exclusively cytoplasmic (e.g. androgen and glucocorticoid receptor), both cytoplasmic and nuclear (e.g. mineralocorticoid receptor and thyroid hormone

receptor B), and exclusively nuclear (estrogen receptor α , progesterone receptor) (Chopin-Delannoy et al. 2003). For instance, glucocorticoid receptors (GRs) present in the cytoplasm are translocated into the nucleus after binding to a hormone, while thyroid receptors (TRs) are localized in the nucleus regardless of the presence of a ligand.

For EcR and its heterodimer USP, we still do not have a clear knowledge of their distribution patterns and the mechanism controlling that. The investigation from the Lammerding-Koppel group in *C. tentans* showed that the distribution of EcR and USP was more like that of other homodimerizing steroid hormone receptors. The immunohistochemical work within epithelial cell line from *C. tentans* demonstrated that EcR and USP were present in both nuclei and in granular compartments of the cytosol in an untreated cell line. However, when treated with 20E, both EcR and USP shifted from cytoplasm to nucleus (Lammerding-Koppel et al. 1998). This observation was supported by the study in mammalian COS-7, CHO-K1 and HeLa cells, where fused protein of EcR and fluorescent pore is expressed. Note that fused USP showed an exclusively nuclear distribution in transfected cells, which was different from previous observations (Nieva et al.

2005). The subsequent research in mammalian cell lines showed that muristerone A increased nuclear localization of EcR (Nieva et al. 2007; Nieva et al. 2008). Stocker et al. demonstrated that ecdysteroid drove EcR to locate at the specific ecdysteroid-induced puff-sites on chromosomes in the salivary gland of *C. tentans* (Stocker et al. 1997). Since ligand binding increased the affinity of the EcR-USP heterodimer to 20E-response elements (Koelle et al., 1991; Oro et al., 1990; Yao et al., 1993), it is reasonable to conclude that ligand binding to EcR facilitates the transportation of EcR from cytoplasm to nucleus. However, this paradigm is not consistent with the mode of action of classic heterodimer nuclear receptors in vertebrates, whose EcR-USP is hypothesized to bind to its target genes in the apo-conformation (Mangelsdorf and Evans 1995). This hypothesis was further tested by Hu et al. who showed in their experiments that gene repression and activation were regulated by apo- and holo-EcR dimerization of EcR and USP with alternative partners (Hu et al. 2003).

Further research provided more evidence in receptor shuttling theory. Tomasz et al. discovered the subcellular trafficking of EcR and USP was determined by their nuclear localization and nuclear export signals (NLSs and NESs). USP has only

NLSs, whereas EcR has both NLSs and NESs (Gwozdz et al. 2007). So, the explanation of nuclear receptor shuttle between the cytoplasm and the nucleus is acceptable (Black et al., 2001; Bunn et al., 2001; Prufer and Barsony, 2002; Shank and Paschal, 2005). Intracellular localization is the result of a dynamic shuttling of receptor proteins, which are imported continuously by interaction with DNA or the nuclear matrix, for example, retained in the nucleus (Vafoyopoulou 2009), and are exported to various degrees. It has been confirmed that both import and export of EcR, USP and EcR-USP are energy dependent events (Betanska et al. 2007). Export of EcR and EcR-USP is mediated by exportin-1 (CRM-1), while no export signal could be identified for USP. It is also demonstrated that the export signal of EcR is impaired by dimerization that renders the nuclear export signal located in the ligand binding domain of EcR inaccessible. Thus, EcR and USP can enter the nucleus independently and the intracellular localization is regulated individually for each receptor.

Elmogy and colleagues have recently observed that membrane-associated EcR might be involved in the transmission of nongenomic signals elicited by ecdysteroids (Elmogy et al. 2004). Thus, in contrast to the traditional static view,

EcR and USP appear to be the key components of a dynamic network that triggers genomic and nongenomic action in different compartments of a cell (Schlattner et al. 2006).

1.3.5 Coregulators and chaperones

The functions of a ligand–receptor complex are usually regulated by coregulators (or cofactors), which can be generally divided into two categories, coactivators and corepressors. Ligand (hormone) binding leads receptors to dissociate from corepressors and associate with coactivators, which, in turn, activate or repress specific gene transcription. The most well studied corepressor in *D.*

melanogaster is SMRT EcR-cofactor (SMRTER), which carries LXXLL amino acid motifs associated with NR interactions and EcR physical interaction sites (Sedkov et al. 2003). Alien, product of the *alien* gene in the *Drosophila* genome, was characterized as a corepressor for the thyroid hormone receptor. Alien binds EcR and SVP in *D. melanogaster* as well, which implied its corepressor role in 20E-induced gene transcription (Papaioannou et al. 2007). Taiman (TAI), the homologue of a steroid receptor coactivator of p160, has been shown to colocalize

with EcR and USP *in vivo*, and has the ability to evoke an elevated ecdysteroid-inducible transcriptional response in cell culture and coprecipitates with EcR (Bai et al. 2000). TRR is the product of the trithorax-related (*trr*) gene, which has also been reported to be an ecdysone-dependent coactivator in *Drosophila* (Sedkov et al. 2003). Another EcR interacting protein, Rig (rigor mortis), is required as a coactivator for induction of the E74A isoform, which normally appears as ecdysteroid titers increase, though it is not required for E75A, EcR, or USP transcription (Gates et al. 2004).

Besides the coregulators, some molecular chaperones are also involved in ecdysteroid signal transduction (Pratt and Toft 1997; Gehring 1998; Arbeitman and Hogness 2000). Interaction with a molecular chaperone-containing heterocomplex (MCH) is required for the activation of the steroid receptors in vertebrates (Pratt and Toft 1997). MCH contains molecular chaperones (Hsp90 and Hsc70), molecular chaperone interacting proteins (Hop, Hip, and p23), and peptidyl-prolyl isomerases (FKBP51, FKBP52, Cyp-40), which interact with the vertebrate steroid receptors and facilitate the folding of receptors. In insects, a few components of MCH have been identified and shown to be necessary for

ecdysteroid signal transduction. Song et al. (1997) showed that a member of the immunophilin family, FKBP46, associated with the EcR-USP receptor complex in the prothoracic gland of *M. sexta* (Song et al. 1997). Research from the same lab suggested that hsc70 could be involved in a negative feedback loop regulating assembly of the ecdysone receptor complex (Rybczynski and Gilbert 2000). Later, a report from the Hogness group described a complex of six proteins (Hsp90, Hsc70, Hip, Hop, FKBP52, and p23) that were necessary and sufficient to activate the ligand binding ability of EcR-USP complex *in vitro*. Among the six proteins, Hsp90 and Hsc70 are demonstrated to be required for activation of the EcR-USP heterodimer *in vivo* in *D. melanogaster* (Arbeitman and Hogness 2000). Recently, Hsc70 was shown to be involved in 20E signal transduction in *H. armigera* as well (Zheng et al.).

1.3.6 Possible scenarios of 20E mode of action

Ecdysteroid receptor is synthesized at the endoplasmic reticulum in the cytoplasm and eventually resides in the neighborhood of a target gene to control the transcription of this gene. What happens in between these events? Three plausible scenarios were reviewed by Palli et al. (Palli et al. 2005).

Scenario 1

Like any other steroid hormone receptor, EcR is part of a larger complex MCH before it encounters the ligand. This complex is located in the cytoplasm and is required for correct folding of the receptor peptide, for stabilization of the monomeric apo-conformation, and possibly for keeping the unliganded EcR out of action. Once ligand binds to the EcR monomer within the complex's constituents, subsequent steps, including heterodimerization, nuclear translocation, DNA binding, and finally target gene activation, ensues sequentially from ligand binding. Although none of the sites at the EcR's surface with which the complex's components may interact have yet been determined and mapped, a decisive role of chaperones and their release by the action of ligand must be considered as a possibility for EcR activation (Arbeitman and Hogness 2000).

Scenario 2

Similar to scenario 1, EcR is located in cytoplasm and trapped in MCH. However, it is USP rather than ligand that releases EcR from the cytoplasmic complex. The heterodimer of apo-EcR and USP then translocates to the nucleus and binds to

DNA independently of ligand. It is only the very last step (i.e., the activation of AF-2) that requires ligand (20E) binding (Hu et al. 2003). Rather than being the singular general signaling pathway, this second route may be invoked primarily by stress conditions.

Scenario 3

Ligand-induced dimerization with USP causes EcR to leave the cytoplasmic complex (MCH), and then the interaction with USP leads EcR to an intermediate conformation. In this conformation, EcR exhibits an increased affinity with USP (A scenario in which ligand and USP would simultaneously interact with EcR is not considered for probabilistic reasons). The holo-EcR-USP heterodimer translocates to the nucleus, binds to DNA and co-activators, and eventually activates transcriptions of target genes.

1.4 20E-induced gene transcription cascade

The research on polytene chromosomes in *C. tentans* and *D. melanogaster* open a gate to understand the mode of action of ecdysteroids at the molecular level.

Polytene chromosomes are the result of chromosomal replication without mitosis,

which shows characteristic puff patterns in the salivary glands of *D.*

melanogaster and *C. tentans* in certain development stages.

Those puffs are the loci on DNA actively transcribing mRNA. Clever and Karlson

(1960) first showed that injection of 20E into last instars of *C. tentans* induced

chromosomal puffing in 2 hours, and the puff regions are the same as those

observed during normal initiation of pupariation. The research by Ashburner

and his colleagues (1972, 1976) suggested that the puffs were divided into “early”

and “late” puffs. The “early” puffs were activated within 1 hour by adding 20E and

start to regress after 4 hours, while, the “late” puffs started to emerge at 4 hours.

20E could directly induce the “early” puffs without protein synthesis, whereas the

regression of “early” puffs and appearance of “late” puffs need the participation of

new proteins. Later, Ashburner and colleagues also determined that the late puffs

could be divided into two classes, based on their regulation by ecdysone. The

early-late puffs are induced relatively rapidly after the addition of hormone and

require the continuous presence of ecdysone for their activity, much like the early

puffs. The late-late puffs, in contrast, are induced at a later time and are

prematurely induced upon ecdysone withdrawal. This latter result was

interpreted to mean that the ecdysone–receptor complex might exert a direct negative effect on the late-late gene expression.

Molecular studies have provided significant support of the Ashburner model and the model can now be revised to include the word "gene" after "puff". The milestones of this process are the isolation and characterization of the genes responsible for EcR and USP and the following discoveries of ecdysteroid-inducible 'early puff gene' (BR-C, E74, E75) in the early puff position and 'late puff gene' (L72) in the late puff position of the chromosome (Baehrecke 1996). A modified flow chart of the Ashburner model is depicted in **Fig. 8**, along with the two classes of late puff genes that were not included in the original model.

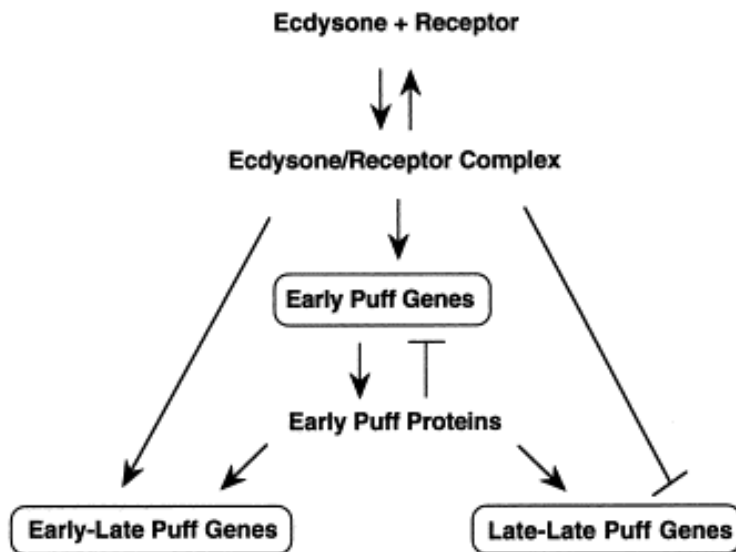


Fig. 8. The modified Ashburner model for ecdysone-regulated puff gene expression. Ecdysone, bound to its receptor, directly induces the early

puff genes. These genes express proteins that perform dual regulatory functions – they repress their own expression and induce the late puff genes. The early-late puff genes are also dependent on direct induction by the ecdysone–receptor complex, resulting in their expression earlier than the late-late puff genes, which are repressed by the ecdysone–receptor complex (Thummel 2002).

1.5 Membrane-bound ecdysteroid receptor

Classically, steroid hormones mediate their actions following the three basic steps,

(1) binding to intracellular receptor; (2) migrating to the nucleus with the help of the receptor; (3) binding to a specific hormone response element and inducing

changes in gene expression. This process is called the ‘genomic response’ of a

hormone, which is a relative slow process, usually 30 minutes to several hours. In

addition to the ‘classical’ genomic response, the steroid hormones can also exert

rapid effects within a short period of time, even in seconds. These responses are

nominated as ‘nongenomic response’ or ‘rapid response’. The reports of

nongenomic response of ecdysteroids in arthropods are summarized in a table by

The shortest response time allowing for nuclear receptor-mediated nongenomic

steroid hormone effects is unknown. It must be less than one second as judged

from observations with a vertebrate cell system (Revankar et al. 2005).

Nongenomic response of ecdysteroid is mediated by a different mechanism from

the genomic response, which is related to receptors binding to membrane.

Elmogly et al. first provided the evidences of the existence of a putative membrane receptor located in *Bombyx mori* anterior silk glands, but they did not identify the receptor in their study (Elmogly et al. 2004). Later, the same group reported that the level of cAMP increased within 30 s after 20E stimulation, which suggested a ecdysteroid regulated cell/tissue response through a cAMP dependent pathway in non- genomic response mode (Elmogly et al. 2006). The first ecdysteroid-response membrane receptor was identified as a membrane-bound G-protein-coupled receptor (GPCR) in *Drosophila*. A neuronally expressed GPCR, namely DmDopEcR, is activated by dopamine and has a high affinity for ecdysteroids. DmDopEcR may represent the *Drosophila* homologue of the vertebrate “gammaadrenergic receptors” and its physiological relevance is still unclear (Srivastava et al. 2005). Coincidentally, in the same year, research in vertebrates showed that GPCR30, an intracellular transmembrane estrogen receptor, may be responsible for the initiation of nongenomic signaling events in human cell lines (Revankar et al. 2005).

Another candidate of nongenomic response receptor of ecdysteroid is EcR.

Schlattner et al. suggested that the nuclear receptor--EcR might have both a

nuclear and a plasma membrane-associated localization as in the case for the nuclear estrogen receptor. EcR was suspected to have two conformational states depending on the situation, which is termed as “conformational compatibility model” (Schlattner et al. 2006). This assumption was also supported by the observation of estrogen receptor in vertebrates (Razandi et al. 2003; Razandi et al. 2004). Novel signaling pathways for membrane-mediated effects of ecdysteroids will likely to be intensively studied in the near future.

1.6 Phosphorylation in steroid hormone signal transduction

Although the primary signal of steroid receptor activation is the binding of ligand, there are other activities involved in the mediation of the cell response to hormone stimulation. Post-translational modifications (PTM) of steroid receptors play significant roles in regulating receptor stability, activity and subcellular localization and so on. PTM refer to the attachment of biofunctional chemical group to amino acids, such as acetate, phosphate, various lipids or carbohydrates, which changes the chemical nature of an amino acid (e.g. citrullination) or make protein structural change, like the formation of disulfide

bridges. Phosphorylation, acetylation, ubiquitylation and sumoylation are the mainly studied PTMs in steroid hormone signal transduction pathways (Faus and Haendler 2006). Among those PTMs, phosphorylation is the most well studied PTM and with wealthy literature data, including some extensive reviews (Weigel 1996; Weigel and Moore 2007; Weigel and Moore 2007).

As mentioned above, all vertebrate steroid hormone receptors are phosphoproteins (Kuiper and Brinkmann 1994; Weigel 1996), which may allow additional fine tuning of the hormonal response by regulation of the activity of the receptor protein by phosphorylation/dephosphorylation at the cellular level. Studies of steroid hormone receptors have shown that phosphorylation can play roles in many ligand induced signal transduction events. In general, phosphorylation can affect the activities of receptor as diverse as protein stability, nuclear localization, sensitivity to hormone, DNA binding and protein-protein interactions which determine the specificity and extent of the regulation of target genes.

1.7 Phosphorylation in 20E signal transduction

Based on the previous research, we have gained a good understanding about the 20E-induced signal transduction pathway on a macro-view level; however, we are still short of the knowledge of this process at a micro-view level. We still do not fully understand how 20E, EcR and USP interact with each other, and the three possible scenarios of ecdysteroid mode of action remained a puzzle to scientists. To clarify this question, we need to seek the aid of the researches in vertebrates. As we mentioned above, numerous studies have been done in vertebrate steroid receptor phosphorylation. Briefly, phosphorylation participates in almost every aspect of a ligand-induced nuclear receptor response, including sensitivity of hormone response, DNA binding, expression, stability, subcellular localization, dimerization, and protein-protein interactions. And this regulation of function is receptor specific, site specific, and often dependent on the cellular context (Ward and Weigel 2009). Therefore, we would expect that phosphorylation also plays roles in the 20E-induced signal transduction pathway.

1.7.1 EcR and USP are phosphoproteins

In order to study the role of phosphorylation in ecdysteroid signal transduction, we need to know whether EcR and its heterodimer are phosphoproteins. Putative phosphorylation sites on USP and EcR were found in many insects, including *A. aegypti*, *B. mori*, *C. tentans*, *Choristoneura fumiferana*, *D. melanogaster*, *Heliothis virescens*, *M. sexta*, *Lucilia cuprina* and *Tenebrio molitor*. Rauch et al. first showed EcR and USP proteins are phosphoproteins under *in vivo* conditions in cells of *C. tentans* (Rauch et al. 1998). Later in the same year (1998), Song et al. demonstrated that in the prothoracic gland of *M. sexta*, a phosphorylation form of USP, p47 is positively correlated with ecdysteroidogenesis. The λ -phosphatase inhibitor was used to show the different phosphorylation levels of USP and EcR (Song and Gilbert 1998). Using an antibody raised against *D. melanogaster* USP, Nocoli et al. discovered USP has five immunoreactive bands in *T. molitor* by Western blot analysis, which corresponds to different phosphorylated forms of USP. The nuclear form of TmUSP seems unphosphorylated. An *in vivo* 20-hydroxyecdysone treatment increases considerably and rapidly the phosphorylated forms of TmUSP (Nicolai et al. 2000). USP was identified as a phosphoprotein in *D. melanogaster* as well. Western blot analysis of USP showed only one band (p54) after adding λ phosphatase inhibitor, while there were two

bands (p56 and P54) in the non-treatment group, suggesting that USP exists in *Drosophila* as a 54kd non-phosphorylated form or a 56kd phosphorylated form (Song et al. 2003).

1.7. 2 20E controlled phosphorylation

Is the phosphorylation of EcR and USP related to 20E stimulation? Some evidence indicates that 20E stimulates the phosphorylation of USP, but not EcR.

In *C. tentans*, the phosphorylation pattern of EcR is not modified, but phosphorylation of USP increases considerably after 20E treatment (Rauch et al. 1998). Similar phenomena were observed in other insects as well. In *T. molitor*, 20E induces a rapid hyperphosphorylation of TmUSP (in 1 h), leading to the accumulation of the most phosphorylated form (51 kDa) 24 h after treatment (Nicolai et al. 2000). In *M. sexta*, Song & Gilbert (1998) showed that 20E, but not ecdysone, was responsible for initiating phosphorylation of USP in the prothoracic gland. In *D. melanogaster*, USP displayed an increasing percentage of the phosphorylated form in dosage and temporal 20E stimulation assays (Song et al. 2003). These data agree well with the numerous studies showing that

steroid receptors become hyperphosphorylated in the presence of hormone or other agonists, and sometimes in the presence of antagonists (for review (Orti et al. 1992)). In conclusion, 20E stimulation could affect the phosphorylation status of USP, usually by hyperphosphorylating USP. However, we do not have direct evidence that EcR phosphorylation status is also affected by 20E stimuli, though EcR is a phosphoprotein.

1.7.3 Phosphorylation regulated by protein kinase C (PKC)

The protein kinase consensus recognition sequence analysis of EcR reveals multiple phosphorylation sites on the EcR sequence, with 10 sites for protein kinase C (PKC), 11 sites for casein kinase II (CKII), and a single site for protein kinase A (PKA); USP has 10 potential phosphorylation sites, with 7 sites for PKC and 4 for CKII (Rauch et al. 1998). Recently, protein kinase C (PKC) mediated phosphorylation of USP has been shown to be a necessary step in 20E-induced gene expression in *D. melanogaster* (Sun and Song 2006). Though USP has both CKII and PKC phosphorylation sites, CKII was not the protein kinase responsible for USP phosphorylation; it was PKC that regulated the phosphorylation of USP.

When PKC activity was blocked by its specific inhibitor chelerythrine chloride (CC), the phosphorylated USP P56 decreased while the P54-unphosphorylated USP form increased accordingly. After PKC was demonstrated to be responsible for USP phosphorylation, it was critical to know whether the PKC-mediated USP phosphorylation played any roles in regulating the expression of 20E-induced genes and proteins. RT-PCR results using salivary glands that were preincubated with the PKC inhibitor chelerythrine chloride followed by 20E challenge showed that chelerythrine chloride inhibited the PKC-mediated USP phosphorylation and thus blocked the transcriptional expression of 20E-induced genes, i.e., the early-puff gene E74A and E75B and the late-puff gene DHR3, E78A, and E78B, but had no effect on the genes that were not induced by 20E under the experimental conditions, i.e., the intermolt puff gene Sgs4, the stage-dependent puff gene FTZ-F1A, and the early-puff gene E74B. These results suggest that PKC-mediated phosphorylation is required for the expression of 20E-induced genes and inhibition of PKC activity results in inhibition of USP phosphorylation and subsequent 20E-induced gene transcription (Sun and Song 2006). Study of receptor of activated C kinase (RACK) in 20E-induced transcription factor CHR3 expression also supported that PKC regulated phosphorylation is necessary for

20E signal transduction. RACK is a helper protein that can bind PKC and function as a shuttling protein to translocate the activated PKC to appropriate subcellular sites of action. The research of RACK in *C. fumiferana* showed that the expression of 20E-induced CHR3 was suppressed when RACK was blocked. This suggests that PKC is a necessary component in the 20E signal transduction pathway. Since phosphorylation may influence the USP distribution in cytoplasm and nucleus, thus, it is one possible interpretation of how PKC regulates 20E signal transduction (Quan et al. 2006).

The PKC superfamily is a family of serine/threonine kinases involved in the regulation of many cell functions. The PKC family constitutes a group of multifunctional Ser/Thr protein kinases involved in metabolism, mitogenesis and gene expressions (Farese 2002; Saito and Shirai 2002). To date, at least 12 isoforms of PKC have been identified in vertebrates, which are divided into three subfamilies: conventional (or classical) PKC, novel PKC and atypical PKC. Depending on mode of activation the PKC family is divided into three subgroups: Conventional isoforms (α , β_1 , β_2 , γ) are dependent on both Ca^{2+} and diacylglycerol (DAG) for stimulation of activity, novel isoforms (δ , ϵ , θ , η) are dependent on DAG, and the atypical isoforms (ζ , ι , N1, N2) are independent of

Ca²⁺ and DAG but are activated by phosphatidic acid and phosphatidylinositol-3,4,5-trisphosphate (PIP₃) (Saito and Shirai 2002). In *Drosophila*, six PKC isoforms have been identified based on the available genomic sequence (Shieh et al. 2002), including two classical PKCs (PKC^{53E} and eye-PKC), two novel PKCs (PKC^{98E} and PKC^{delta}), two atypical PKC (DaPKC and PKC-related kinase N).

1.8 Goals and approaches in this thesis project

Although there is plenty of data showing that PKC plays important roles in 20E signal transduction, little is known about what the exact roles PKC plays in this pathway. To gain a comprehensive understanding of PKC-regulated phosphorylation in 20E signal transduction, we set up the following objectives in our research.

- 1)** To study the relationship between phosphorylation and subcellular localization of USP and EcR.

- 2)** To determine whether PKC-regulated phosphorylation could affect the 20E-induced gene transcriptions at protein level.

3) To determine which PKC isoform(s) is responsible for the regulation of the 20E signal transduction pathway in salivary glands of *D. melanogaster*.

4) To investigate all PKC-regulated phosphorylation involved in the 20E signal transduction.

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CHAPTER 2

Proteomic identification of PKC-mediated expression of 20E-induced protein in *Drosophila melanogaster*

A portion of this work has resulted in the following manuscript:

Sun, Y., S. An, et al. (2007). "Proteomic identification of PKC-mediated expression of 20E-induced protein in *Drosophila melanogaster*." J Proteome Res **6**(11): 4478-88.

2.1 Introduction

Insect steroid hormones, mainly 20-hydroxyecdysone (20E), trigger and coordinate the molting and metamorphosis of insects via the ecdysone receptor (EcR) and its heterodimer ultraspiracle protein (USP) (Riddiford et al. 2000; Henrich 2005). Both EcR and USP are members of the nuclear receptor superfamily and are ligand-activated transcription factors. The activities of nuclear receptors are subjected to regulation not only by transcriptional/translational mechanisms but also by posttranslational mechanisms such as phosphorylation. Studies of transcription factors in vertebrates have shown that phosphorylation can play roles in nuclear

translocation, DNA binding, interactions with other proteins, and transactivation (Weigel 1996; Lange 2004). In insects, both EcR and USP have been demonstrated to be phosphoproteins and EcR and USP phosphorylation are regulated by 20E (Rauch et al. 1998; Song and Gilbert 1998; Nicolai et al. 2000; Song et al. 2003). Phosphorylation of EcR and USP has been shown to play roles in mediating the ligand- and perhaps also DNA-binding activity in the prothoracic glands of *M. sexta*. 20E-induced expression and phosphorylation of a specific isoform of USP in the *Manduca* prothoracic glands has been associated with changes in ecdysteroidogenic activity both *in vivo* and *in vitro*, suggesting a feedback mechanism by which ecdysteroid synthesis is downregulated. Thus, phosphorylation provides another layer of mechanism to regulate the function of the EcR-USP complex.

For studies of ecdysteroid action, the larval salivary gland (SG) of *D. melanogaster* has been extensively studied as a model for unraveling the hierarchy of cellular response to steroid hormones. Briefly, specific genes within these SGs incubated with 20E rapidly undergo changes in transcriptional

activities that are mediated by the functional EcR-USP heterodimer. Later, the transcription of these early genes regresses as the transcription of late genes is induced by early gene products (Thummel 2002). Recently, protein kinase C (PKC) activity has been shown to be necessary for USP phosphorylation in the larval SG of *D. melanogaster*. Inhibition of PKC activity by a PKC-specific inhibitor, chelerythrine chloride (CC), inhibits transcription of early 20E-induced genes, including E74A, E75B, DHR3, and the late genes, including E78A and E78B. Nevertheless, inhibition of PKC activity does not affect other genes that are not induced by 20E (Sun and Song 2006). The involvement of a PKC signaling pathway in 20E-induced gene transcription is further supported by its requirement for the receptor of activated C kinase 1 (RACK1), a shuttling protein that translocates the activated PKC to appropriate subcellular sites for target protein phosphorylation (Ron et al. 1999; Rigas et al. 2003). RACK1 is necessary for 20E-induced expression of the transcription factor CHR3 in the spruce budworm *C. fumiferana* (Quan et al. 2006). Although EcR and USP phosphorylation has been implicated in mediating the ligand- and DNA-binding activities of the EcR-USP complex and PKC has been shown to be involved in 20E-induced gene transcription, little is known about the roles of PKC in

mediating the subcellular translocation of EcR and USP and 20E-induced protein expression in *D. melanogaster*. In the present study with the larval SG of *D. melanogaster*, several novel 20E-induced proteins have been identified using a proteomic approach. The transcription of genes encoding these proteins display characteristics of both early and late responsive genes, and their induction is reduced by inhibiting PKC activity. Further, PKC activity plays a role in mediating subcellular translocation of *D. melanogaster* EcR and USP, suggesting a mechanism for at least some of these transcriptional outcomes.

2.2 Materials and Methods

Insects

D. melanogaster (wild type^{ore}) was reared on artificial blue diet (Fisher Scientific, catalog no. 22315) at 24°C under constant darkness. The blue diet allows easy synchronization of the early wandering third instar larvae based on the protocol described by Andres and Thummel (Andres and Thummel 1994), and the synchronized early wandering third instar larvae were used for the following experiments.

Immunohistochemical localization of EcR and USP in PKC inhibitor-treated glands

To investigate the effect of PKC-specific inhibitor chelerythrine chloride (CC) on EcR and USP subcellular localization, SGs from early wandering larvae were dissected under Ringer's solution (3.6 mM NaCl, 54.3 mM KCl, 8.0 mM CaCl₂, and 28.3 mM MgCl₂) and immediately placed in a 12-well tissue culture plate containing 500 µL of Grace's insect tissue culture medium. After dissection, the medium was carefully removed from the well and replaced with 500 µL of fresh Grace's medium containing the indicated concentrations of PKC-specific inhibitor CC and incubated for 6 h at room temperature. The control group was incubated with Grace's medium only. For study of temporal response, SGs were incubated with 100 µM CC for 3, 6, 12, and 24 h.

After incubation, the glands were fixed in freshly prepared 4% paraformaldehyde for 2 h and washed thoroughly with phosphate-buffered saline (PBS) (136 mM NaCl, 1.1 mM K₂HPO₄, 2.7 mM KCl, 8.0 mM Na₂HPO₄, pH 7.4) for 15 min. The fixed SGs were permeabilized in PBS containing 0.5% Triton-100 for 40 min,

then preincubated in a blocking solution (5% bovine serum, 5% goat serum in PBS) for 2 h at room temperature. The primary antibody was prepared either at a 1:200 dilution for AB11 USP-specific monoclonal antibody (mAb) or a 1:100 dilution for DDA 2.7 EcR mAb in 1:10 diluted blocking solution. The permeabilized SGs were incubated with either EcR or USP primary antibody overnight at 4 °C, washed three times with PBS for 15 min the following day, and then incubated with secondary antibody (goat antimouse-Alexa Fluor 568) at a 1:400 dilution in a 1:10 diluted blocking solution for 2 h. After briefly washing with PBS, SGs were incubated with SYTOX Green nucleic acid stain (Molecular Probes, catalog no. S7020) for 15 min followed by three washes with PBS before being mounted on coverslides with Aqueous Mounting Medium (Permafluor). EcR and USP signals were observed with a BioRad confocal system (BioRad Radiance 2000) at the Molecular Cytology Core of the University of Missouri–Columbia.

Western blot analysis of the effect of CC on EcR and USP distribution in cytosolic and nuclear fractions

SGs of early wandering third instar larvae were dissected and cultured in Grace's medium containing 100 μ M PKC inhibitor CC for 6 h at room temperature. The control group received Grace's medium only (CC was dissolved in water). After incubation, cytosolic and nuclear proteins were prepared from the SGs according to a modified protocol by Alnemri et al. (Alnemri et al. 1993). Briefly, SGs from each treatment were collected into a 1.5 mL tube, respectively, washed with fresh Grace's medium twice, and resuspended in 100 μ L of cell lysing buffer (50 mM HEPE, pH7.4, 1 mM PMSF, 1% NP40). After 10 min incubation on ice, the SGs were centrifuged at 1000*g* for 10 min and supernatant was collected as cytosolic protein preparation. The pellet was briefly rinsed with the cell lysing buffer to remove remaining cytosolic proteins and added with 100 μ L of nuclei extraction buffer (20 mM HEPES, pH 7.4, 5 mM KCl, 0.5 mM DTT, 0.5 mM MgCl₂, 0.6 M NaCl). Following incubation on ice for 15 min with occasional vortex, the sample was centrifuged at 16000*g* for 15 min. Supernatant was collected as soluble nuclei protein fraction, and the pellet was briefly rinsed with the nuclear lysing buffer and collected as the nuclear pellet.

Protein concentration was determined using the BioRad protein bioassay kit (reagent A: catalog no. 500-0113; reagent B: catalog no. 500-0114), and protein samples were subjected to SDS-PAGE and Western blot analysis using AB11 USP mAb (1:1000) and DDA 2.7 EcR mAb (1:500), respectively, as described previously.

2D gel analysis of 20E-induced proteins in PKC inhibitor-treated glands

SGs from early wandering third instar larvae were dissected in Ringer's solution and preincubated in Grace's medium for 30 min in the presence or absence of 100 μ M PKC inhibitor CC and then stimulated with or without 0.5 μ M 20E (a predetermined effective dose) for 6 h. At the end of incubation, the SGs were collected, homogenized, and centrifuged at 16000g for 10 min. Protein concentration in the resulting supernatant was quantified as described above.

The resulting protein samples from each treatment were subjected to 2D gel electrophoresis and Coomassie blue staining as described previously. In brief, the first dimensional isoelectric focusing (IEF) was performed using BioRad Protein

IEF cell and ReadyStrip IPG strips (pH 3–7, 11 cm, BioRad catalog 0165-4000) according to the manufacturer's instructions. Each IPG strip was soaked in a rehydration buffer containing 100 µg of total protein per treatment. After isoelectric focusing, the IPG strip was equilibrated and subjected to SDS-PAGE (8–16% Tris-HCL gradient gel, BioRad catalog 0345-0105) and Coomassie blue (G-250) staining.

In-gel protein digestion

Coomassie blue-stained 2D gels were compared between the treatments, and differentially displayed protein spots were identified. Protein spots that were induced or enhanced by 20E but inhibited by the PKC inhibitor were excised from the 2D gel using a 1.5 mm diameter spot picker (The Gel Company, Catalog#P2D1.5). The excised gel pieces were subjected to in-gel digestion according to the standard protocol provided by the MU Proteomics Center [modified from Havlis et al. (Havlis et al. 2003) and Jiménez et al. (Jimenez et al. 2001)]. Briefly, the excised gel pieces were moved to clean 1.5 mL Safe-Lock eppendorf tubes (Eppendorf, catalog no. 022363204), destained three times with

500 μ L of 50/50 (v/v) acetonitrile/100 mM ammonium bicarbonate solution for 15 min at RT with agitation, and washed briefly in 500 μ L of acetonitrile. Gel pieces were then dehydrated for 20 min with 500 μ L of acetonitrile (RT, with agitation) and rehydrated for 2 h at 4°C in 5 μ L of a 20 μ g/mL solution of modified TPCK-treated porcine trypsin (Trypsin Gold, mass spectrometry grade, 17000U/mg, catalog no. V5280, Promega, Madison, WI) in 40 mM ammonium bicarbonate/10% acetonitrile. Subsequently, the trypsin solution was replaced with 15 μ L of 40 mM ammonium bicarbonate/10% acetonitrile, and the proteins were digested overnight at 37 °C. The digests were acidified by addition of 4 μ L of extraction solvent and were transferred into 500 μ L tubes. Each gel piece was extracted twice with 10 μ L of 600/300/100 (v/v/v) acetonitrile/water/10% trifluoroacetic acid solution for 10 min with gentle agitation at RT. Extracts from the same sample were pooled, snap frozen in liquid N₂, and stored at -80°C. The freezing extractions were dried by lyophilization and redissolved in 5 μ L of 0.1% TFA in 5% acetonitrile. The rehydrated samples were then concentrated and desalted using Eppendorf Perfect pure C18 tips and eluted in 5 μ L of 0.1% TFA in 50% acetonitrile following the manufacturer's instructions.

MALDI-TOF spectrometry of tryptic digests

The digested and ZIP-tipped samples were spotted onto a stainless-steel MALDI plate with an equal amount of CHCA matrix (5 mg/mL in 60% ACN, 0.3% TFA, 10 mM ammonium phosphate). An equal volume (0.5 μ L) of CHCA matrix was added and allowed to crystallize at room temperature. A peptide mass spectrum was acquired using an Applied Biosystems 4700 proteomics analyzer, which was operated in a positive ion mode, and spectra were acquired over a mass range of 800 to 4000 Da. Peptide calibration standards (4700 calibration mix, Applied Biosystems) were used to calibrate the instrument in MS mode using six peptides of known mass. Calibration was achieved by the “plate model and default” mode for MS of six external calibrant spots. MS/MS calibration was also conducted using fragment ions of the 1570.7 Glu-1-fibrinopeptide B on all six calibrant spots. MS acquisition was conducted over an 800 to 4000 Da mass range. Internal MS calibration using at least two trypsin autolysis peptide ions was also done when these ions were present (above signal/noise ratio of 10). Mass fingerprints were processed with the software GPS Explorer (version 3.6). Ions with S/N ratios > 20 (excluding trypsin autolysis) up to a maximum of 65 per spectrum were submitted for search for protein matches against the NCBI nr database limited

to *D. melanogaster* (last updated September 17, 2006) using the “combined MS and MS/MS” function of the GPS Explorer software. Search parameters allowed one trypsin miss-cleavage and the following modifications to peptides: carbamidomethyl cysteines and methionine oxidation.

Characterization of the matched protein candidates using real-time PCR and Western blots

SGs were dissected and preincubated with 100 μ M PKC inhibitor CC for 30 min and then stimulated with 0.5 μ M 20E for 6 h, the same conditions noted for 2D gel analysis. For real-time PCR measurements, samples were collected at 2 and 6 h. After incubation, total RNA was isolated from each treatment using Trizol reagent (Invitrogen) according to the manufacturer's instructions, and RNA concentration was determined by measuring the absorbance at 260 nm on a spectrophotometer. The extracted total RNA was treated with RNase-free DNase I (Promega, catalog no. M6101) to eliminate potential contamination by genomic DNA. About 1.5 μ g of total RNA from each sample was used for the first-strand cDNA synthesis. The first-strand cDNA synthesis was primed using oligo (dT)

based on the SuperScript First-Strand synthesis kit (Invitrogen, catalog no. 11904-018). The synthesized cDNA was used as template for estimation of gene transcription in SGs by real-time PCR. The 20E responsive genes and the corresponding primers used in real-time PCR are listed in **Table 1**.

primer name	forward	reverse
1. GD	5'-GAGGACTACAAGAACGAGCAC-3'	5'-GAATGACCAGGATGTTGCGAT-3'
2. ATPase B	5'-GTCCGTGCTGGATGACCACAC-3'	5'-GTCCGTAAGGATGACCAGCA-3'
3. FBP1	5'-AACCTGCCACCCTTAGCG-3'	5'-TGCTGGACTCCGAAAGTGC-3'
4. LP07910	5'-CGTTAGCGTGAACAGTGAC-3'	5'-TGCTGGACTCCGAAAGTGC-3'
5. mATP synthase B	5'-CAGAAGCGTTCACCGTCGCT-3'	5'-GCAACAGGGACATCTGACG-3'
6. GA 17461	5'-GCCAACTTCTCAAGGTCGTGTC-3'	5'-GCCGTCCATCTGGTTGAGCAG-3'
7. eIF-4a	5'-GTCGCAAGGTGGACCAACTG-3'	5'-ACCTTGCGACCGAAACGA-3'
8. LD22255	5'-CCACCCGTCTGGGAATCTC-3'	5'-ACTTGGTGAGGAGTAGCA-3'
9. ATPase E	5'-CGAGGTCACCAAGAATCAG-3'	5'-GCCTTGTATTGCTCCACAG-3'
10. RACK1	5'-TTTGTCGGTTGCCTTCTCG-3'	5'-TCCAGACCTTGACGGTGGAT-3'
11. Annexin B	5'-CCATTGCCAGTTCTACGA-3'	5'-AAGGTGGACTCATCTGTGC-3'
12. GST	5'-TACGGCAAGACCGACTCCCTGTA-3'	5'-CACCAGGGCAATGTCGGCTAC-3'
13. LP02306p	5'-GTTATTGGCCGTTTCGTGGT-3'	5'-TGGTTGCTGTTCTCCGCTG-3'
14. HADH	5'-AACATCAATACCGTGGGCACGT-3'	5'-CCCTGGGTGCTCAAGTCAC-3'
DHR3 (+ ctl)	5'-CCGCTCAAGGGCGATGTCA-3'	5'-AAACGACATTCGGGTGCTG-3'
FTZ-F1A (- ctl)	5'-CGGAGCGGTCGTGCCACAT-3'	5'-ACCAGCATTCTATCCGCTCGA-3'
Rp49 (CK)	5'-TACAGGCCCAAGATCGTGAA-3'	5'-GACAATCTCCTTGCGCTTCT-3'

Table 1. Real-Time PCR primers for verifying the identified proteins.

Real-time PCR amplification and analysis were carried out on an Applied Biosystems 7500 fast real-time PCR system (ABI). The final volume of reaction was 25 μ L using ABI SYBR Green Supermix (ABI). For real-time PCR, the reaction was held at 95°C for 10 min followed by 40 cycles at 95°C for 15 s, then 60°C for 1 min. The specificity of the SYBR Green PCR signal was confirmed by melting curve analysis and agarose gel electrophoresis. The mRNA expression was quantified using the comparative CT (cross threshold, the PCR cycle number

that crosses the signal threshold) method (Livak and Schmittgen 2001). The CT of the housekeeping gene rp49 was subtracted from CT of the target gene to obtain Δ CT. The normalized fold changes of the target gene mRNA expression were expressed as $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT$ is equal to $\Delta CT_{\text{treated sample}} - \Delta CT_{\text{control}}$.

Protein was extracted from the SGs preincubated with the PKC inhibitor CC for 30 min and stimulated with 20E for 6 h, the same conditions noted for 2D gel analysis. Polyclonal antibodies against ATPase subunit B (1:1000) (from M. Huss, University of Osnabruck, Osnabruck, Germany) and FBP1 (1:1000) (from Jean-Antoine Lepasant, University of Paris, Paris, France) were used to verify translational expression of ATPase subunit B and FBP1 by Western blot as described above.

2.3 Results

Effect of PKC inhibitor on EcR and USP subcellular translocation

To investigate the effect of CC, a PKC-specific inhibitor, on EcR and USP subcellular translocation, SGs from early wandering third instar larvae were incubated for 6 h with the indicated concentrations of PKC-specific inhibitor CC

and immunostained with EcR (DDA2.7) and USP (AB11) mAb, respectively. In freshly dissected SGs and in SGs incubated for 6 h in the absence of CC, both EcR and USP signals were localized primarily in SG cell nuclei (**Fig. 9.**). However, when the SGs were incubated for 6 h with the PKC inhibitor, both EcR (**Fig. 10a.**) and USP (**Fig. 10b.**) nuclear signals declined in a dose-dependent manner starting at 25 μM CC. At 50 μM , most EcR and USP signals disappeared and at 100 μM or higher, EcR and USP signals were barely visible. The trace amount of the remaining EcR signal was mainly localized at the periphery of the nucleus, while the residual USP signal was more evenly distributed in the nucleus. Temporal response studies revealed that both EcR and USP signals started to decline when the SGs were incubated with 100 μM PKC inhibitor for 3 h and became barely detectable when the SGs were incubated with the PKC inhibitor for 6 h or longer (data not shown).

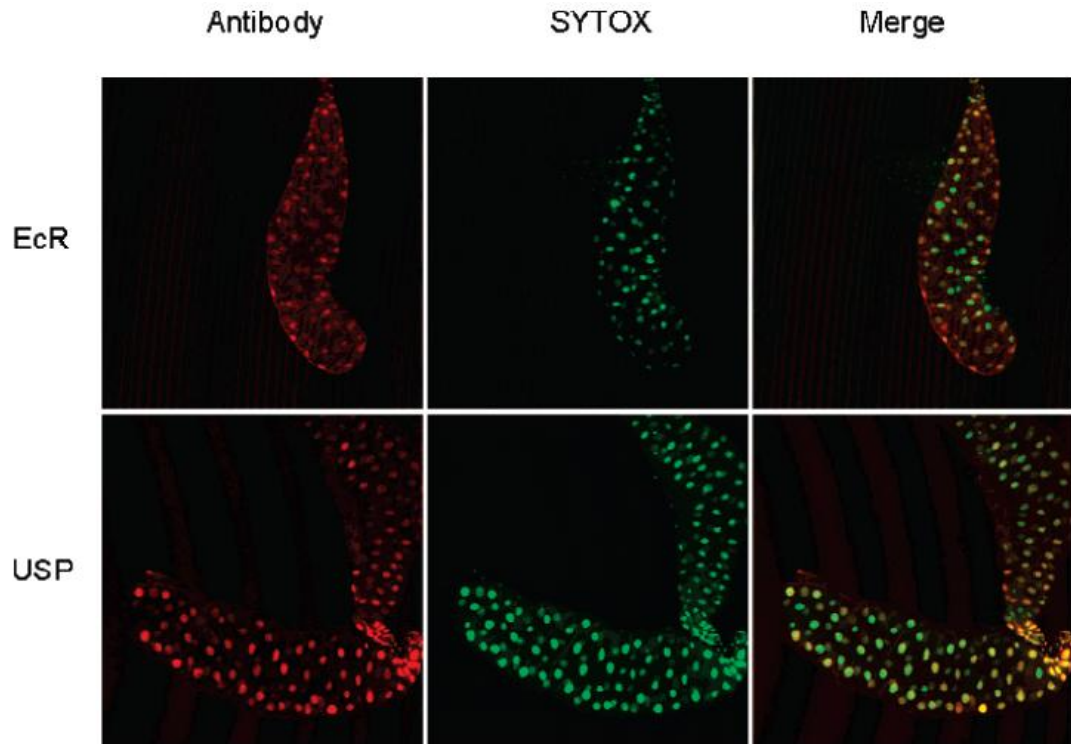


Fig. 9. Immocytochemical localization of EcR and USP. Salivary glands of early wandering larvae of *D. melanogaster* were dissected in Grace's medium and immunostained with EcR (a) or USP (b) mAb (red color) and then counterstained with SYTOX Green nucleic acid stain (green color). EcR and USP signals were visualized using a confocal microscope (BioRad Radiance 2000) at the MU Cytology Core.

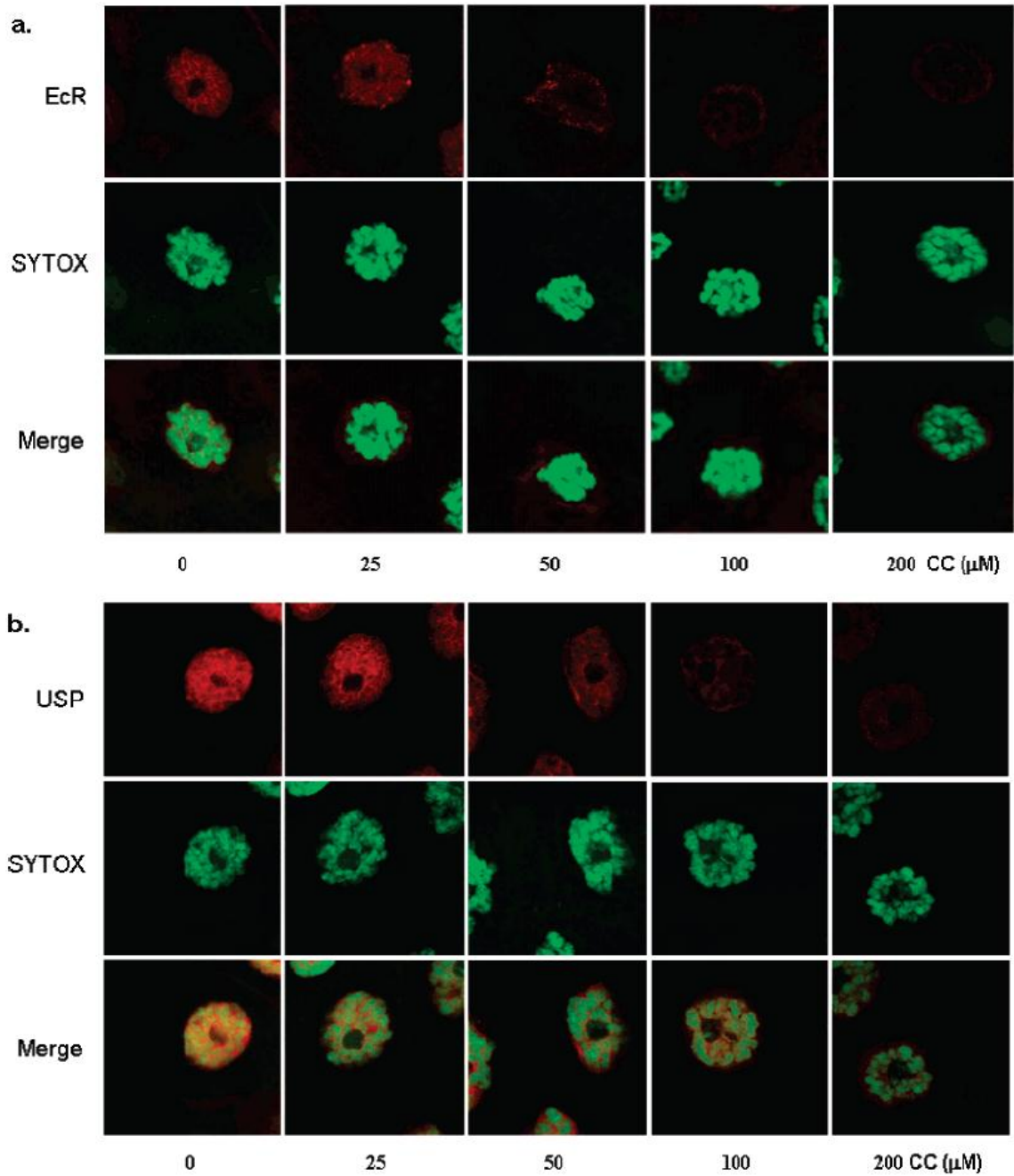


Fig. 10. Effects of the PKC-specific inhibitor chelerythrine chloride (CC) on EcR and USP subcellular localization. Salivary glands from early wandering third instar larvae were incubated for 6 h in Grace's medium containing the indicated concentrations of CC and immunostained with EcR or USP mAb (red color). Nucleus of the glands was stained with SYTOX Green nuclei acid stain (green color). EcR (a) and USP (b) signals were visualized using

a confocal microscope (BioRad Radiance 2000). At least 3–5 biological replicates were performed, and the images are typical.

To investigate whether the disappearance of EcR and USP signals from the nucleus of the PKC inhibitor-treated SGs (**Fig. 10a, b**) was caused by the inhibition of EcR and USP protein expression by the inhibitor, the SGs were incubated for 6 h in the presence or absence of 100 μ M PKC inhibitor and lysed with specific buffer to obtain cytosolic and nuclear preparations. The latter were further treated with the nuclear lysing buffer to separate the soluble nuclear fraction from the insoluble nuclear pellet. The resulting samples were analyzed by Western blot using the aforementioned EcR and USP specific antibodies. As shown in **Fig. 11.**, nearly all the signal of EcR was located in the nucleus in the absence of the PKC inhibitor, with approximately 40% in the soluble nuclear fraction (**Fig. 11.** top panel, lane 2) and the rest in the insoluble nuclear pellet (**Fig. 11.** top panel, lane 3) while about 50% USP was in the cytosol (**Fig. 11.** bottom panel, lane 1), and the rest appeared in the soluble nuclear fraction (**Fig. 11.** bottom panel, lane 2). There was no detectable USP signal in the insoluble nuclear pellet (**Fig. 11.** bottom panel, lane 3). As expected, both phosphorylated and nonphosphorylated forms of USP were detected in both the cytosolic and the

soluble nuclear fraction (**Fig. 11.** bottom panel, lanes 1 and 2). Phosphorylated and nonphosphorylated forms of USP in *Drosophila* SGs have been previously distinguished using a lambda protein phosphatase treatment assay followed by Western blot analysis.

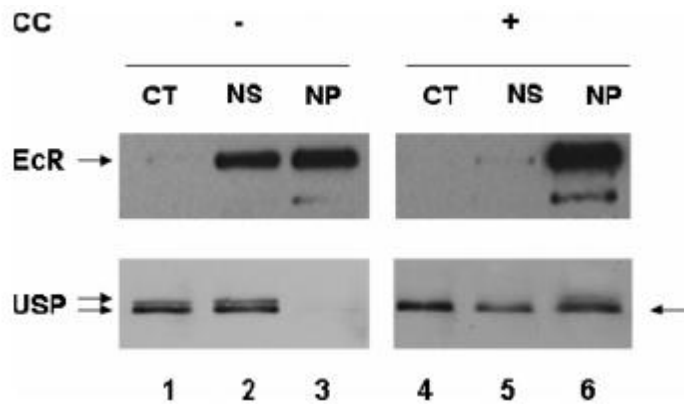


Fig. 11. Western blot analysis of the effects of PKC inhibitor CC on EcR and USP subcellular distribution. Salivary glands of *D. melanogaster* from early wandering third instar larvae were incubated for 6 h in Grace's medium containing 100 μ M CC. Cytosolic and nuclear fractions were subjected to SDS-PAGE (denatured) and Western blot analysis using EcR and USP mAb. At least 3–5 biological replicates were performed, and the presented images are typical. CT: cytosol. NS: soluble nuclear fraction. NP: insoluble nuclear pellet.

When the glands were incubated with the PKC inhibitor, nearly the entire EcR signal in the soluble nuclear fraction disappeared (**Fig. 11.** top panel, lane 5) while the EcR signal in the nuclear pellet doubled (**Fig. 11.** bottom panel, lane 6), suggesting that PKC inhibitor causes EcR to shift from the soluble nuclear

fraction to the insoluble nuclear pellet. In the presence of PKC inhibitor, the phosphorylated USP form disappeared while the nonphosphorylated USP signal increased accordingly (**Fig. 11.** bottom panel, lanes 4 and 5). The PKC inhibitor blocked USP phosphorylation and caused the shift of more than half of the USP signal from the soluble nuclear fraction to the insoluble nuclear pellet, but did not cause the shift of USP from the cytosol to the nuclear fractions. These results suggest that the PKC inhibitor does not affect the expression of EcR and USP proteins, a result that confirms a previous report (Sun and Song 2006). Nevertheless, CC affects the solubility of EcR and USP in the nucleus.

Effect of PKC inhibitor on 20E-induced protein expression

To examine whether the alteration of EcR and USP cellular solubility by the PKC inhibitor inhibited 20E-induced protein expression, SGs from early wandering larvae were preincubated with 100 μ M PKC inhibitor for 30 min and then challenged with 0.5 μ M 20E for 6 h. This incubation period is sufficiently long to allow 20E-induced changes in expression that allow for the recovery of 20E-induced proteins and sufficiently short to minimize the appearance of proteins

resulting from secondary cellular changes that are not directly associated with 20E action.

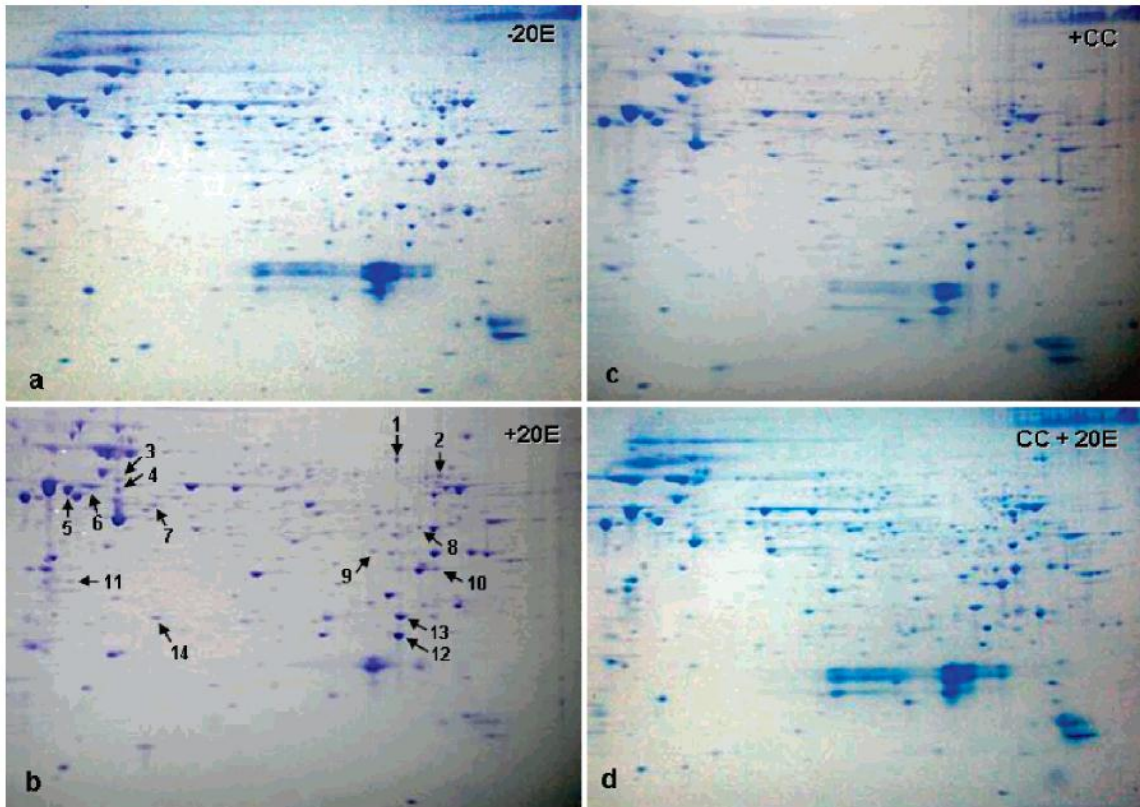


Fig. 12. 2D gel analysis of PKC inhibitor CC-mediated expression of 20E-induced proteins. Salivary glands from early wandering third instar larvae of *D. melanogaster* were preincubated for 30 min in the absence (a and b) or presence (c and d) of 100 μ M CC and then challenged for 6 h without (a and c) or with (b and d) 0.5 μ M 20E. Proteins were extracted from each treatment and subjected to 2D gel electrophoresis and Coomassie blue (G250) staining. The proteins induced by 20E and blocked by CC were labeled with numbers. A minimum of three biological replicates for each treatment were performed.

At the end of the incubation, proteins were extracted from the glands and subjected to 2D gel electrophoresis, followed by Coomassie blue staining. 2D gel maps revealed approximately 41 differentially expressed protein spots: 21 induced and 20 inhibited by 20E (**Fig. 12a, b** not labeled). Only 14 out of the 21 proteins induced by 20E were inhibited by the additional presence of CC (**Fig. 12b, d**). Major protein spots induced by 20E and inhibited by CC were labeled with numbers and excised for protein identification.

Protein identification

20E-induced protein spots whose induction was inhibited by CC treatment were trypsinated and subjected to MALDI-TOF MS/MS analysis. The resulting MS fingerprinting data were used to search for protein matches in the NCBI nr protein databank. As shown in **Fig. 13a**, all 14 proteins had positive match-ups in a protein databank with MASCOS scores ranging from 82 to 715 (MASCOS score > 60 represents significant, $p < 0.05$). These proteins can be roughly grouped into seven broad categories based on protein function, including protein actions, lipid metabolism, signal transduction, cell protection, cell functions, storage protein,

and energetics/metabolism. The relative induction ratio of these proteins by 20E ranged from 1- to 5-fold with 5-fold representing newly induced proteins (**Fig. 13b**), and the percent of inhibition of these proteins by CC ranged from 30 to 100% (**Fig. 13c**) when the protein spots in the Coomassie blue-stained 2D gel maps were analyzed on the basis of their relative density using an AlphaImager digital system. Five out of the 14 proteins were newly induced by 20E, including RACK1, annexin B9a, 3-hydroxyacyl-CoA dehydrogenase, glutamate dehydrogenase, and LD22255p (**Fig. 13b**). Their 20E-induced expression was completely blocked by CC, except for RACK1, which was only partially inhibited (approximately 60%; **Fig. 13c**). The expression of the nine other characterized proteins was modestly affected by 20E (0.74- to 3.8-fold), and in all cases, the additional presence of CC resulted in a partial reduction of quantitative protein levels (25–80%).

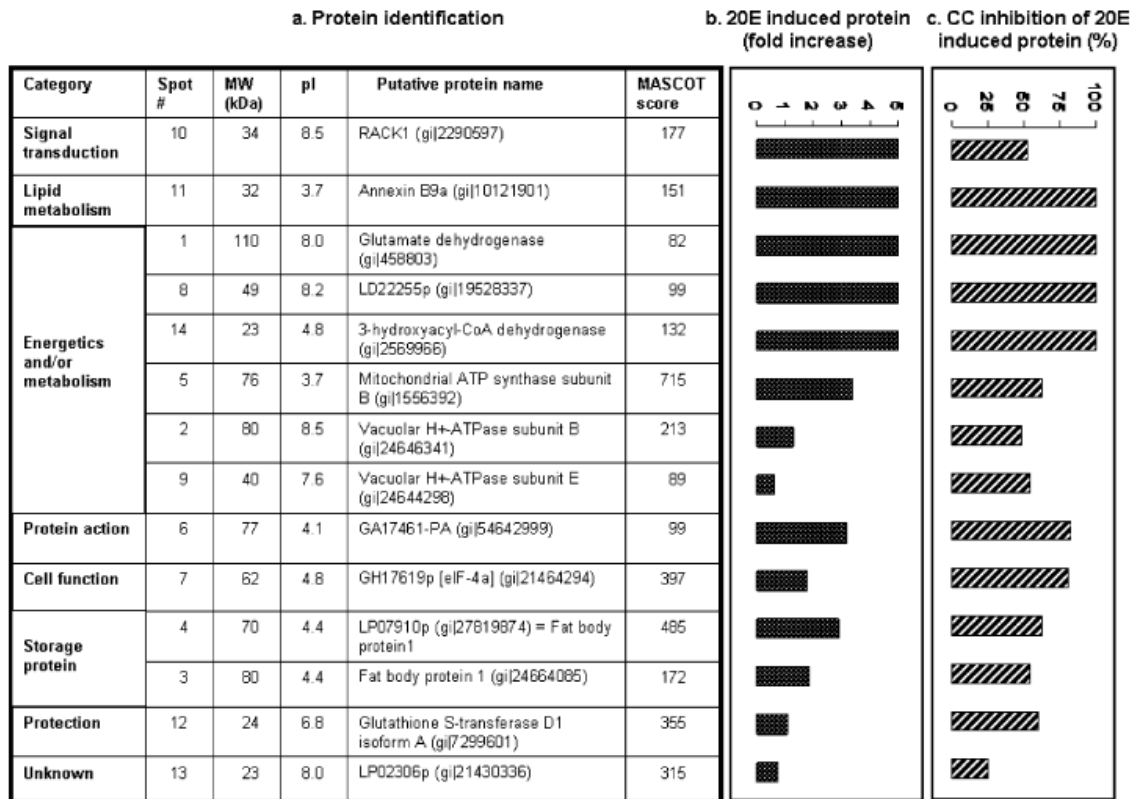


Fig. 13. A list of proteins induced by 20E and inhibited by simultaneous CC treatment. 20E-induced and CC-blocked proteins were subjected to MALDI-TOF MS/MS analysis, and the resulting MS/MS fingerprinting data were used to search for matches against the NCBI database. The identified proteins are listed in (a). MASCOT scores > 60 are significant ($p < 0.05$). The ratio of induction by 20E (b) and inhibition by CC (c) are also given. The data represent the mean \pm SE of three biological samples.

Transcriptional regulation of the identified proteins

The classic action of 20E involves its interaction with the EcR-USP heterodimer, which in turn affects the transcriptional activity of target genes. To confirm that the quantitative increases in protein levels registered on 2D gels (**Fig. 12**) follow

from changes in transcriptional activity associated with 20E, we designed primers (**Table 1.**) to all 14 proteins on the basis of a protein databank search of MALDI-TOF MS/MS data. To assess whether the changes involved early transcriptional changes that might be direct targets of ecdysteroid receptor function or later transcriptional changes that result indirectly from early changes, we examined the transcript levels after 2 and 6 h incubations with 20E using quantitative real-time PCR (**Fig. 14.**) and/or Western blot (**Fig. 15.**). As a control for this analysis, the early response gene, DHR3, was selected, since its promoter is a direct target of the EcR-USP heterodimer and its induction is robust (Hiruma and Riddiford 2004). The induction of DHR3 by 20E is completely offset by the addition of the PKC inhibitor, suggesting that the phosphorylation state of EcR and/or USP is important for its regulation. Transcript levels of a second early inducible gene, E74A, was increased by about 70-fold with 20E (data not shown). E74A is strongly induced by 20E titers of approximately 10^{-7} M and higher (Karim and Thummel 1991). As shown in **Fig. 14**, transcripts for all 14 proteins could be grouped into four categories on the basis of their response to 20E stimulation: (1) early response, (2) late response, (3) early suppressed, and (4) late suppressed.

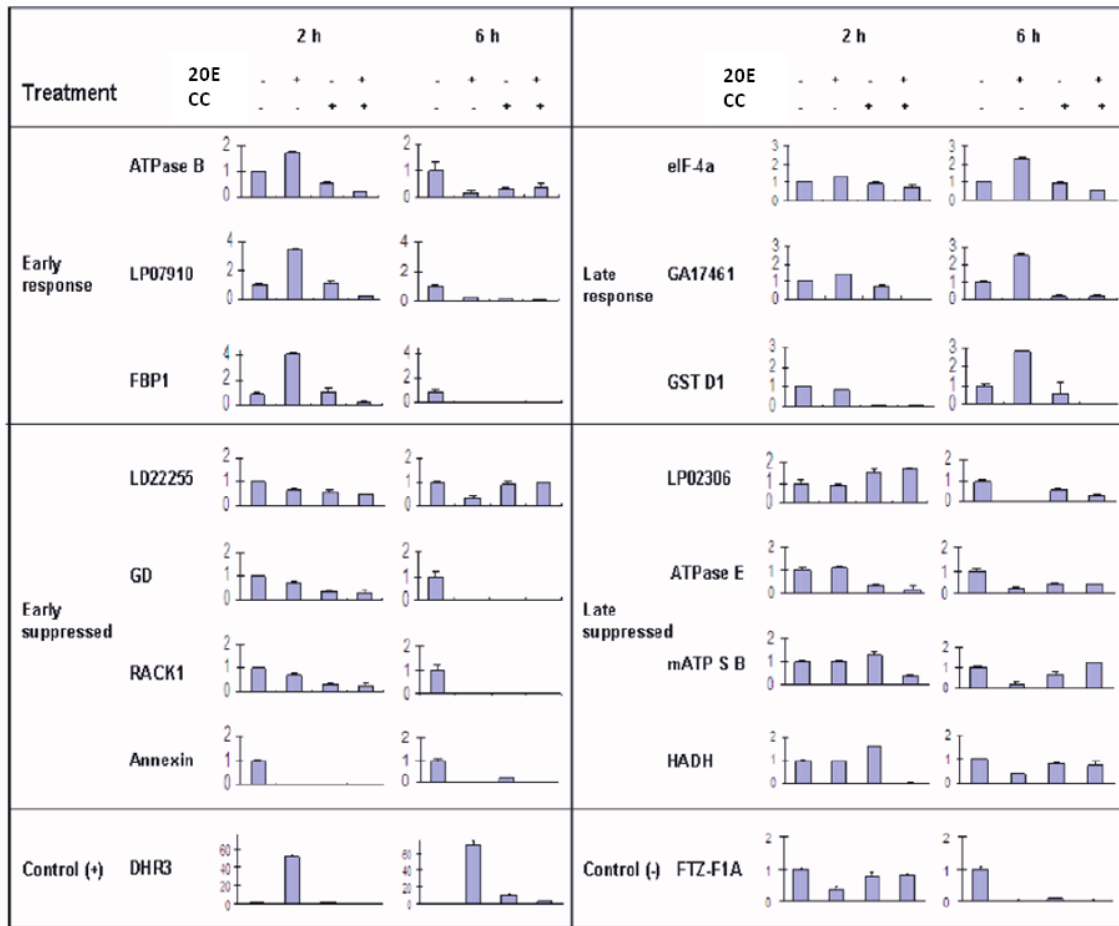


Fig. 14. Real-time PCR analysis of transcription for genes encoding identified proteins. The SGs from early wandering third instar larvae of *D. melanogaster* were preincubated for 30 min in the presence or absence of 100 μ M CC and then challenged for 2 and 6 h with or without 0.5 μ M 20E. Total RNA was isolated for real-time PCR analysis of transcription levels for all 14 identified proteins. DHR3 was used as a positive control for 20E induction and FTZ-F1A as a negative control. The rp49 gene was used for normalization of the compared templates. The data represent the mean \pm SE of three biological samples.

For early response genes, transcripts of vacuolar H⁺-ATPase subunit B, FBP1, and LP07910 were induced by 20E by 1.9- to 4.0-fold at 2 h, and all were inhibited by

the additional presence of CC. Further, the transcription of these genes was suppressed by 20E after 6 h, consistent with the patterns predicted for early response genes. Interestingly, CC not only eliminated 20E induction but reduced transcript levels below the basal level in other control groups, suggesting that phosphorylation plays a role not only for increasing transcript levels but also for regulating basal transcript levels. In the late response category, 20E had no significant effect on the transcript levels of eIF-4a, GST, and GA17461-PA at 2 h, but transcript levels were increased by 2.2- to 2.8-fold at 6 h. Again, the PKC inhibitor completely blocked 20E-induced gene transcription at 6 h.

Simplistically, it follows that increases in protein levels correspond to increases in transcript levels after 2 and/or 6 h of 20E treatment. Paradoxically, transcript levels that are suppressed by 20E and/or PKC-inhibitor treatment are associated with some of the proteins whose levels were increased by a 6 h 20E treatment. In the early suppressed category, transcripts of LD22255, GD, RACK1, and annexin B9a were significantly inhibited by 20E at 2 h and nearly completely blocked at 6 h. PKC inhibitor had no effect on 20E-suppressed gene transcription except that CC restored LD22255 mRNA to basal level at 6 h (see Discussion). For the late

suppressed genes, 20E had no significant effect on mRNA levels of mitochondrial ATP synthase subunit B, vacuolar H⁺-ATPase subunit E, 3-hydroxyacyl-CoA dehydrogenase, and LPO2306 at 2 h, but inhibited transcription of these genes at 6 h. The PKC inhibitor blocked basal level transcription of mitochondrial ATP synthase subunit B, vacuolar H⁺-ATPase subunit E, and 3-hydroxyacyl-CoA dehydrogenase at 2 h incubation in the presence of 20E, but not LPO2306, which was enhanced by the PKC inhibitor. Interestingly, the PKC inhibitor was able to restore transcription of these genes to a basal level except ATPase subunit E (see explanation in Discussion) at 6 h, although this effect did not depend upon 20E.

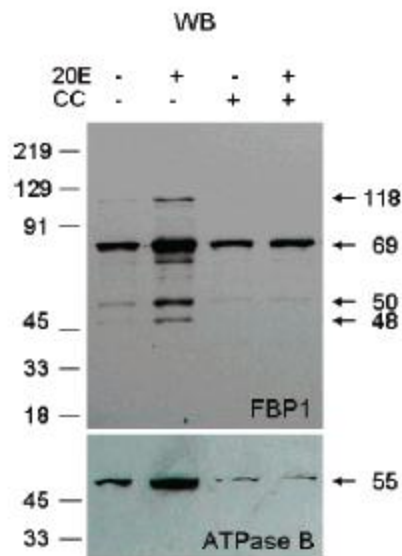


Fig. 15. Western blot verification of two identified proteins. The SGs were treated for 6 h as described for RNA extraction and subjected to denaturing SDS-PAGE and Western blot analysis using polyclonal antibodies against FBP1 (top panel) and ATPase subunit B (bottom panel).

Western blot analysis was also performed to verify ATPase subunit B and FBP1 using antibodies available for these proteins. As shown in **Fig. 15**, both ATPase subunit B and FBP1 were induced by 20E, and this response was blocked by PKC inhibitor, a result that corresponded well with the expression of ATPase subunit B and FBP1 at the protein level as registered in 2D gel maps (**Fig. 12**). Western blot analysis of FBP1 (**Fig. 15a** top panel) showed four identical FBP1 bands reported by Burmester et al. (Burmester et al. 1999), which were all induced by 20E and blocked by CC, further confirming the 2D gel (**Fig. 12**) and real-time PCR data (**Fig. 14**). Similarly, Western blot analysis of ATPase subunit B revealed that it was induced by 20E, but that its expression was completely blocked by CC (**Fig. 14b** bottom panel) and actually fell below the basal level. In other words, the protein levels were consistent with those seen in 2D gel maps (**Fig. 12**), but negatively correlated to the mRNA level (**Fig. 14**).

2.4 Discussion

PKC has previously been demonstrated to play a role in mediating the transcriptional regulation of the 20E-induced early puff gene E74A and E75B and

the early late puffs, DHR3, E78A, and E78B, via the EcR-USP complex. It has no effect on genes that are not induced by 20E in late larval glands, i.e., the intermolt puff gene Sgs4, the stage-dependent puff gene FTZ-F1A, and E74B, which is normally inhibited by the 20E titers. In the present study, we investigated how PKC regulates EcR and USP subcellular localization and 20E-induced protein expression. Using CC, we showed that inhibition of PKC by its specific inhibitor resulted in the decline of EcR and USP signals in the nucleus of salivary glands in a dose- and temporal-dependent manner (**Fig. 10**).

By itself, this finding suggests that inhibition of PKC activity by CC inhibits the expression of USP and EcR protein, increases the degradation of EcR and USP protein, or both. We speculated that the diminishing USP and EcR signals in the CC-treated glands, as shown in immunocytochemical staining (**Fig. 10**), could result from the inaccessibility of the antibodies to the EcR-USP complex caused by CC treatment under the nondenatured conditions needed for immunocytochemical staining. This prompted us to analyze EcR and USP signals in different fractions of cell lysate using the denatured SDS-PAGE and Western blot. Western blot analysis revealed that CC had no effect on total EcR and USP protein contents, a result consistent with our previous report (Sun and Song

2006), but caused a shift of both EcR and USP from the soluble nuclear fraction to the insoluble nuclear pellet (**Fig. 11**). Combining the immunocytochemical staining data with Western blot results, it is reasonable to conclude that disappearance of USP and EcR signals from the nucleus of the PKC inhibitor-treated cells is caused by a change in the solubility of the EcR and USP complex. Moreover, it is conceivable that the transcriptional activity of USP and EcR could be affected by phosphorylation. For instance, preventing phosphorylation of USP could prevent it from fulfilling its normal role as a dimer partner for EcR, thus leading to a loss of 20E inducibility. The same might be true for EcR too. Since protein consensus recognition sequence analysis of *Drosophila* EcR reveals multiple phosphorylation sites for PKC (Rauch et al. 1998). It is likely that PKC is also the key kinase responsible for EcR phosphorylation. Unfortunately, we do not have a good antibody to track the PKC-mediated EcR phosphorylation patterns as we did for USP.

Alternatively, shift of the EcR-USP complex from the soluble nuclear fraction to the insoluble nuclear pellet when phosphorylation is blocked by CC might in turn

indicate that the EcR-USP dimer forms a large complex with nonphosphorylated chaperone proteins. As demonstrated by Arbeitman and Hogness (Arbeitman and Hogness 2000), a functional EcR-USP complex requires six chaperone proteins. In vertebrates, prior to exposure to steroid, some steroid receptors are associated with a chaperone protein complex anchored by HSP90. Upon binding to its cognate ligand, the steroid receptor dissociates from its chaperone complex, undergoes a conformational change to an active state, and ultimately regulates transcription in collaboration with a variety of transcriptional coregulatory factors (Pratt and Toft 1997; Cheung and Smith 2000). Steroid receptor activity, therefore, is regulated not only by the level of hormone but also by the levels/activity of coactivators that are recruited to target promoters to remodel chromatin and facilitate transcription. Many steroid receptors and their coregulators are phosphoproteins whose activities are regulated by diverse cell signaling pathways (Weigel 1996; Weigel and Zhang 1998; Rowan et al. 2000; Lopez et al. 2001; Wu et al. 2004). On the basis of the information forthcoming from this study and results obtained with EcR and other nuclear receptors, we speculate that the chaperone proteins form a complex with the EcR-USP dimer. Upon steroid hormone stimulation, the chaperone proteins are phosphorylated

by protein kinases, and thereby released from the complex, thus activating receptor-mediated gene transcription. This possible mechanism bears some resemblance to the relationship of the vertebrate progesterone receptor (PR) and its chaperone proteins. Upon exposure to progesterone, the ligand-activated PR undergoes a conformational change, dissociates from chaperone proteins, dimerizes, and interacts with specific progesterone response elements in the promoter regions of target genes. The evidence from the present study illuminates the possibility that the EcR-USP complex is activated via phosphorylation of chaperone proteins. Likely, a regulatory relationship exists between nuclear sublocalization and the regulation of nuclear localization by EcR and USP, which has not yet been elucidated (Nieva et al. 2005).

It is critical to know whether the PKC-mediated USP phosphorylation and EcR-USP subcellular distribution ultimately affects the expression of 20E-induced proteins that are inhibited by CC addition to a medium containing 20E. The selected protein spots for MALDI-TOF MS/MS analysis were the major spots visible in Coomassie blue-stained gels, and in most cases, their expression has

not previously been reported as 20E-dependent. Previously identified genes whose transcriptions were induced by 20E and blocked by CC, including E74A, E75B, DHR3, E78A, and E78B, were not among the 14 identified proteins. The possible explanation for this is that the translational expression products of these five genes exist at trace levels and were invisible or barely visible in Coomassie blue-stained gels. Moreover, while the induction of these proteins is robust, the relative molar levels of these proteins is low and possibly temporally transient, since all are associated with early transcriptional response, and normally this response has regressed by 6 h after the initiation of treatment.

The biggest challenges for proteomic identification of the target proteins are to validate them as true targets of 20E action and to confirm their identity immunologically. On the basis of the response of transcripts to 20E incubation for 2 and 6 h, transcripts of the 14 genes can be roughly grouped into four categories: (1) early response, (2) late response, (3) early suppressed, and (4) late suppressed. For early and late response genes, CC inhibited 20E-induced transcription of all six genes (**Fig. 14**), a result that correlated well with the

protein level registered in 2D gel maps and implicated them as direct targets of EcR-USP action (**Fig. 12**). ATPase subunit B and FBP1, two of three early response gene products, were also verified by Western blotting because of the availability of antibodies (**Fig. 15**). These results suggest that PKC-mediated phosphorylation is required for 20E-induced expression of early responsive genes at both transcriptional and translational levels. The ability to block 20E-induced DHR3 expression further implicates USP as either a direct or indirect target of CC action for offsetting the 20E induction of DHR3 and the other early genes, although this inference will require further testing. It is less likely that the EcR-USP heterodimer is related to the effects upon late response genes described here, thus highlighting the likelihood that CC, while specific in its molecular action, affects the transcriptional capabilities of a range of factors. In fact, the ability of CC to eliminate basal transcription belies the likelihood that other transcription factors are affected by this treatment. Moreover, the lack of correspondence for many cases, in which transcript levels are suppressed although the proteins were originally designated by their increased presence, further suggests that CC affects mechanisms associated with mRNA stability, translation, protein stability, and other processes that are not transcriptional.

The effect of 20E on synthesis of chromosomal and cytosol proteins in imaginal discs of *Drosophila* was reported over 25 years ago (Hill et al. 1982), but the identities of these proteins have not yet been revealed. Three of the proteins revealed by this study have been recently reported to be involved in a steroid signaling pathway. For example, RACK1 is a homologue of the G protein β subunit and typically contains seven internal Trp-Asp 40 (WD40) repeats (Ron et al. 1994). This protein appears to be involved in at least three signal transduction pathways: the PKC pathway (Ron et al. 1995; Ron et al. 1999), the cAMP-specific phosphodiesterase PDE4D5 pathway (Yarwood et al. 1999), and the tyrosine kinase–phosphatase pathway (Chang et al. 1998; McCahill et al. 2002). Because RACK1 interacts with several different cellular proteins associated with several signal transduction pathways, it may play a critical role in cross-talk among different signal transduction cascades (McCahill et al. 2002; Rigas et al. 2003). Annexin B9a is another example of a 20E-inducible gene product (Tsuzuki et al. 2001). FBP1 is expressed in the fat body. The appearance of FBP1 in SGs probably resulted from the incomplete removal of the fat body, which is attached to the SGs of early wandering larvae during the dissection. Nevertheless, FBP1 is an abundant protein that is also a direct target of EcR-USP action

(Antoniewski et al. 1994), and its appearance fortuitously confirms the proteomic approach as a basis for examining 20E action and the influence of PKC on the transcription of the genes encoding these proteins.

In *D. melanogaster*, six PKC isoforms have been identified on the basis of the available genomic sequence (Shieh et al. 2002), including two classical PKCs (PKC^{53E} and eye-PKC), two novel PKCs (PKC^{98E} and PKC delta), an atypical PKC (DaPKC), and a PKC-related kinase. DaPKC is essential for early embryonic development, and Eye-PKC plays a role in the regulation of visual signaling, a G-protein coupled phospholipase C beta-mediated cascade. No information is available on which PKC isoform is responsible for USP or EcR phosphorylation. Identification and characterization of the PKC isoform responsible for EcR and USP phosphorylation is currently underway.

In summary, the present study provides evidence that PKC mediates 20E-induced gene expression at both transcriptional and translational levels in a model system for examining steroid hormone action, the larval SG

of *D. melanogaster*. Further, the recovery of known and novel early gene targets of the functional ecdysteroid receptor, as well as the effects of PKC inhibition on the subcellular localization of the receptor's partners, EcR and USP, suggests that the PKC-mediated phosphorylation is tied to their regulation of the 20E response.

2.5 Supplemental material- Apoptosis in 20E treated salivary gland

20E has been shown to induce the programmed cell death (PCD) of larval tissue in insect metamorphosis (Lockshin and Williams 1965; Weeks and Truman 1985), including the SG of *D. melanogaster* (Lockshin and Zakeri 1994). The PCD of the SG of *D. melanogaster* has been confirmed to go through the apoptosis pathway in response to the pulse of 20E (Jiang et al. 1997). In our experiments, 20E was used to challenge SGs in different time periods from 3 to 24 h. In order to avoid the false results from apoptosis induced by 20E, we need to know whether our experiment materials (SGs) are still physiologically normal to respond to 20E stimulation, but not in an apoptotic process as is the case in our immunostaining, 2D gel electrophoresis and RNAi experiments. Here, we used a Terminal

Transferase dUTP Nick End Labeling (TUNEL) assay to examine apoptosis in the 20E treated SGs in different time periods. The TUNEL assay is a method used to detect DNA degradation in apoptotic cells because one of the hallmarks of late stage apoptosis is the fragmentation of nuclear chromatin, which results in a multitude of 3'-hydroxyl termini of DNA ends. This property can be used to identify apoptotic cells by labeling the DNA breaks with fluorescent-tagged deoxyuridine triphosphate nucleotides (F-dUTP). The enzyme terminal deoxynucleotidyl transferase (TdT) catalyzes a template-independent addition of deoxyribonucleoside triphosphates to the 3' hydroxyl ends of double- or single-stranded DNAs and generates DNA strands with exposed 3'-hydroxyl ends. Addition of the deoxythymidine analog 5-bromo-2'-deoxyuridine 5'-triphosphate (BrdUTP) to the TdT reaction serves to label the break sites. Once incorporated into the DNA, BrdU can be detected by an anti-BrdU antibody using standard immunohistochemical techniques. Non-apoptotic cells do not incorporate much of the F-dUTP because of absence of exposed 3'-hydroxyl DNA ends.

Material and Methods

The TUNEL assay was performed using the DeadEnd Fluorometric TUNEL System (Promega Cat.# G3250) with the following modifications. The SGs treated with 20E in different time periods were transferred to 0.01% polylysine-coated slides, fixed with 4% methanol-free formaldehyde solution, and fluorescence stained following the procedure of the DeadEnd fluorometric TUNEL system. SGs were rinsed with PBS twice and then permeabilized with 0.2% Triton X-100 solution in PBS for 5 minutes at room temperature. The SGs were washed with PBS twice and covered with an equilibration buffer (from the kit) for 10 minutes at room temperature. The equilibration buffer was removed, and a reaction buffer containing the equilibration buffer, nucleotide mix, and terminal deoxynucleotidyl transferase enzyme was added to the SGs and incubated at 37°C for 1 h, avoiding exposure to light. To stop the reaction, SGs were incubated for 15 minutes at room temperature with 2x standard saline citrate (SSC), washed with PBS three times, and then stained with propidium iodide solution (Sigma Cat.# P4170) for 15 minutes at room temperature in the dark to label the nuclei of SGs. The slides were washed 3 times, 5 minutes each in PBS and covered with glass coverslips. The samples were analyzed using a BioRad confocal system (BioRad

Radiance 2000) at the Molecular Cytology Core of the University of Missouri–Columbia.

Results

In **Fig. 16**, after 24 hours 20E treatment, no apoptotic signal was detected in the SGs. At 48 hours, TUNEL-positive signal was observed in the SGs treated with 0.5 μ M 20E, but not in control SGs. This result indicated that 0.5 μ M 20E could induce PCD of the SGs at a time between 24 to 48 h in vitro, but not before 24 h. Thus, in our experiments, there was no interference coming from 20E-induced apoptosis when the SGs were incubated with 20E for 2-6 h.

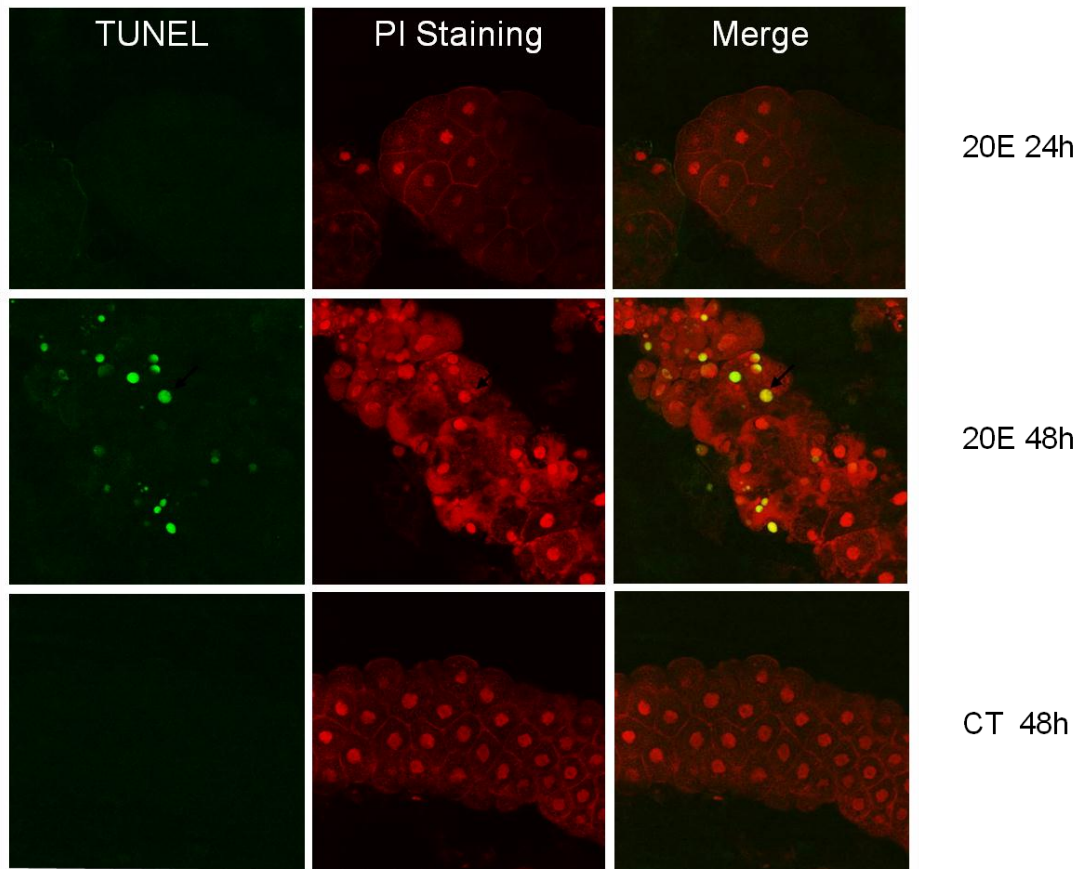


Fig. 16. Detection of apoptosis in the 20E-incubated salivary glands (SG) of *D. melanogaster*. SGs were fixed and processed for dual immunofluorescence to investigate the 20E-induced apoptosis using a TUNEL detection system. The nuclei were stained with the red Alexa Fluor 567 (PI) or the apoptotic signal detected using a green fluorescence in isothiocyanate (FITC) (TUNEL) fluorescence.

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CHAPTER 3

Identification of phospho-PKC substrates in 20E-stimulated salivary gland of *D. melanogaster*

3.1 Introduction

Protein phosphorylation is one of the fundamental mechanisms in regulation of signal transduction pathways. Protein kinases, the enzymes catalyzing protein phosphorylation, are among the largest classes of genes in most organisms, having over 600 members in humans and over 250 members in the fruit fly (Morrison et al. 2000; Manning et al. 2002). Protein kinase C (PKC) belongs to the AGC [cAMP-dependent protein kinase (PKA)/ protein kinase G/ protein kinase C (PKC)] protein kinase family, which is involved in receptor desensitization, regulating cell growth, regulating transcription, mediating immune responses, modulating membrane structure events, and influencing learning and memory. We have shown PKC-mediated phosphorylation is involved in 20E-induced molting process, affecting 20E-induced gene expression at both the transcriptional and translational levels (Sun and Song 2006; Sun et al.

2007). EcR and USP in the ecdysteroid receptor complex are PKC substrates, whose phosphorylation level increased with 20E stimulation (Rauch et al. 1998; Sun and Song 2006). However, we speculate USP and EcR are not the only proteins phosphorylated by PKC in the 20E signal transduction, and we anticipate finding more PKC substrates phosphorylated in this process. To test our conjecture, we need a method to identify as many as possible phosphorylated PKC substrates. In a PKC involved process, the specificity of signal transduction depends on the precise phosphorylation at particular sites of specific PKC substrates. An *in vitro* study of synthetic peptides indicated a preferred PKC consensus phosphorylation motif: RXXS/TXRX (X standing for any amino acid) (Pearson and Kemp 1991). Later, antibodies against specific motifs on phosphorylated PKC substrates were developed by Cell Signaling Technology (Catalog#2261), which enable identification of phosphorylated PKC substrates during different situations. Taking advantage of these antibodies, we investigated all phosphorylated PKC substrates in 20E signal transduction, which adds us more details of the role of PKC-regulated phosphorylation in the 20E-induced molting process. In the present study, we examined the activity of PKC under stimulation

of 20E and also identified several PKC substrates using the phospho-PKC substrate antibody.

3.2 Materials and Methods

Animal rearing and tissue dissection

D. melanogaster (wild type^{ore}) was reared on artificial blue diet (Fisher Scientific, catalog no. 22315) at 24 °C under constant darkness. The blue diet allows easy synchronization of the early wandering third instar larvae based on the protocol described by Andres and Thummel (Andres and Thummel 1994). The synchronized early wandering third instar larvae were used for the following experiments. Salivary glands (SG) from the third instar larvae were dissected under Ringer's solution and immediately placed into a 12-well tissue culture plate containing 500 µL of Grace's insect tissue culture medium. After dissection, the medium was replaced with 500 µL of fresh Grace's medium in the presence or absence of 1 µM 20E and incubated for 10 min, 30 min and 60 min respectively.

After incubation, 70 pairs of SG were collected into 1.5 ml Eppendorf tubes for each treatment and frozen at -80°C for future 2D Western blot analyses.

Reagents

Grace's medium was obtained from GibcoBRL (Grand Island, NY). Phospho-(Ser) PKC Substrate Antibody (catalog# 2261) was ordered from Cell Signaling Technology (Beverly, MA). SuperSignal West Pico Chemiluminescent substrate for Western blot analysis was ordered from Pierce (Rockford, IL). Horseradish peroxidase-conjugated goat anti-rabbit secondary antibody was from Jackson ImmunoResearch Laboratories (West Grove, PA). ReadyStrip IPG strips (pH 3–7, 11 cm, BioRad, catalog# 0165-4000), 2D precast gel (8–16% Tris-HCL gradient gel, BioRad catalog 0345-0105) and Bio-Rad protein bioassay kit (Reagent A, catalog # 500-0113. Reagent B, catalog # 500-0114) were ordered from BioRad (Hercules, CA). Other chemical reagents used for buffers, sample preparation, tissue culture, electrophoresis, and Western blot were obtained from Sigma (St Louis, MO), Fisher Scientific (Houston, TX) or BioRad.

2D electrophoresis and Western blot analysis

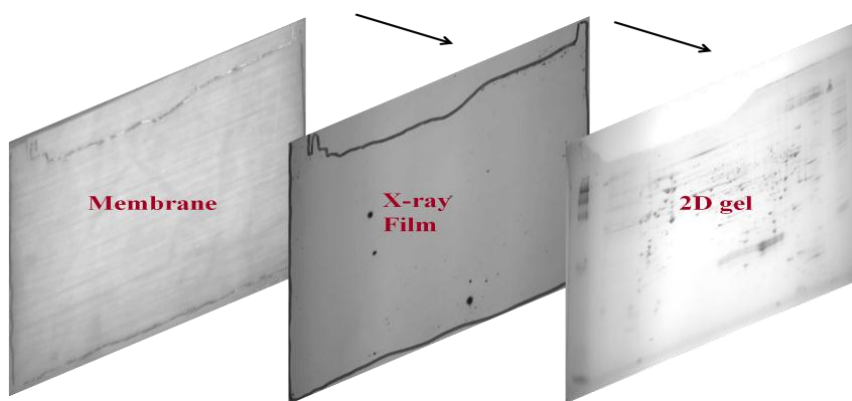
Seventy pairs of SGs from each treatment were homogenized on ice in a phosphate-buffered saline (PBS) (136 mM NaCl, 1.1 mM K₂HPO₄, 2.7 mM KCl, 8.0 mM Na₂HPO₄, pH 7.4) using a disposable pestle in an Eppendorf tube. The homogenate was centrifuged at 16,000 *g* for 10 min at 4 °C to remove debris. Protein concentration in the supernatant was determined using the Bio-Rad protein bioassay kit following the manufacturer's instruction. The resulting protein samples from each treatment were subjected to 2D gel electrophoresis as described previously (Chapter 2). In brief, the first dimensional isoelectric focusing (IEF) was performed using BioRad Protein IEF cell and ReadyStrip IPG strips according to the manufacturer's instructions. Each IPG strip was soaked in a rehydration buffer containing 150 µg of total protein per treatment. After isoelectric focusing, the IPG strip was equilibrated and subjected to SDS-PAGE on a precast 8–16% Tris-HCL gradient gel. Prestained protein molecular weight (MW) standards (BioRad, catalog# 161-0324) were used to monitor electrophoresis and for MW calibration.

After SDS-PAGE, Western blot was employed using phospho-(Ser) PKC substrate antibody. Gel-separated protein samples were transferred onto PVDF

membranes in a transferring buffer (gel running buffer containing 15% methanol) for 30 min at 24 V with a BioRad Trans-blot SD Semidry Transfer Cell. After transfer, the gels were removed from membranes and stained with Coomassie blue (G-250) staining to aid in protein spot identification. The PVDF membranes were blocked with 5% nonfat dried milk in a Tris-buffered saline (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.1% Tween 20, pH 7.5) (TBST) for 1 h, and then incubated overnight at 4°C with Phospho-(Ser) PKC substrate antibody (1:1000) diluted with blocking solution. After incubation with the primary antibody, the membranes were washed three times with TBST for 10 min each, and then incubated with horseradish peroxidase-conjugated goat anti rabbit secondary antibody (diluted at 1:5000 with blocking solution) for 2 h at room temperature. After three 10-minute washes with TBST, the membranes were treated for 1 min with SuperSignal West Pico chemiluminescent substrate (PIERCE, Catalog#34080) and the immunoreactive proteins were visualized by exposing an X-ray film to the membrane.

Protein spot identification and in-gel protein digestion

The X-ray films with Western blot signals were compared between the 20E treatment and control, and differentially displayed signals were labeled. By overlapping the images of X-ray film, PVDF membrane and Coomassie blue stained 2D gel together, we identified the protein spots on the 2D gel corresponding to phosphorylated PKC substrate signals (**Fig. 17**). To be noticed, in our experiments, we used the same number to represent the protein spot and Western blot signal at the same position on X-ray films and 2D gels. By overlapping the images of X-ray film, PVDF membrane and Coomassie blue



stained 2D gel together,

Fig. 17. Scheme of the phosphorylated PKC-substrate identification on the 2D gel.

The protein spots were excised from 2D gels using a 1.5 mm diameter spot picker (The Gel Company, Catalog#P2D1.5), then subjected to in-gel digestion according to the standard protocol provided by the MU Proteomics Center as described previously (**Chapter 2**). The digested protein samples were sent to the MU Proteomics Center for MALDI-TOF spectrometry analysis. To confirm our protein identification results in 2D Western blot, we used an online phosphorylation prediction software- NetPhosK- (<http://www.cbs.dtu.dk/services/NetPhosK/>) to predict PKC specific phosphorylation sites of these proteins.

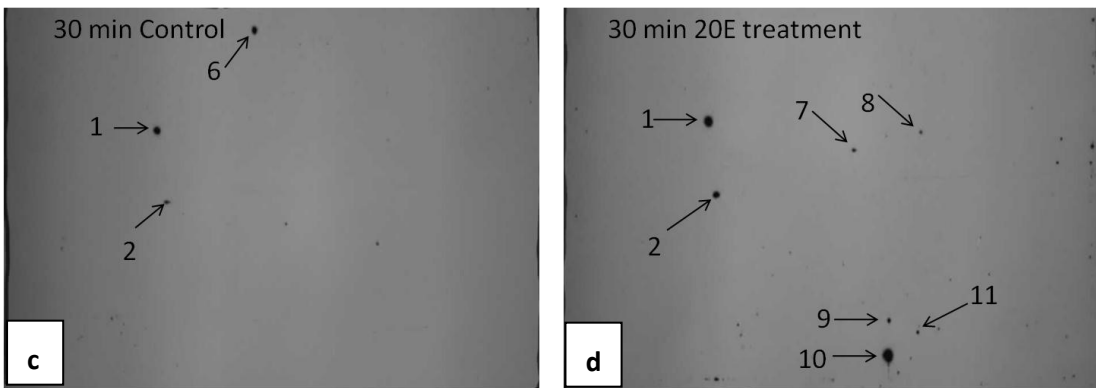
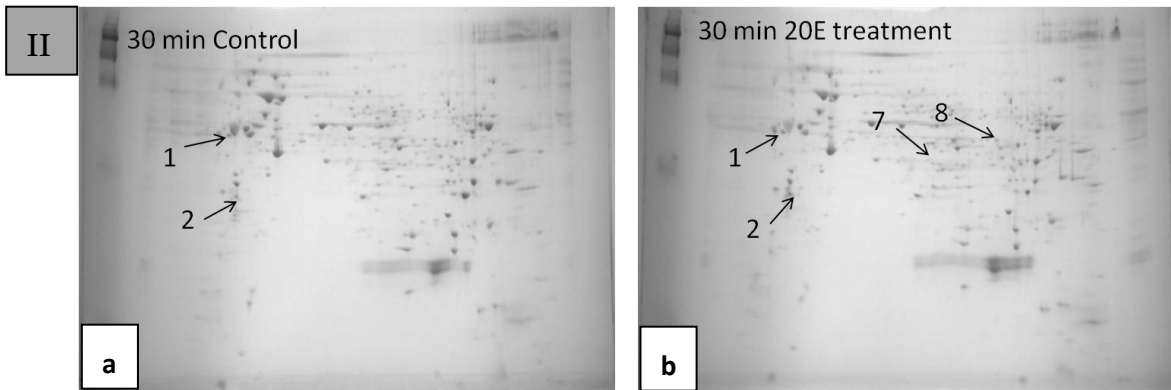
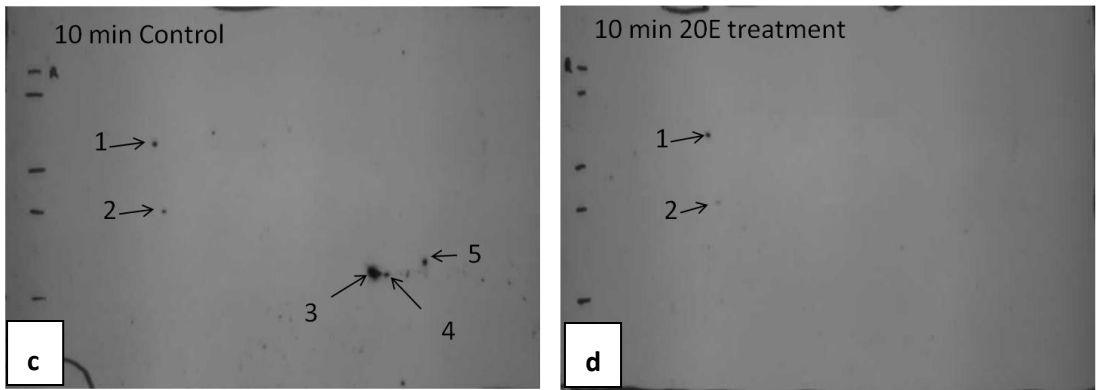
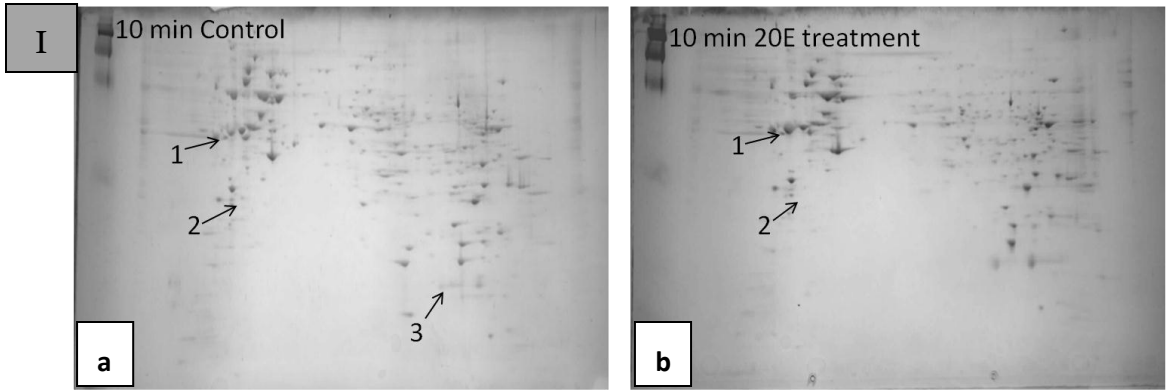
3.3 Results

Identification of PKC substrates by two dimensional Western blot

After 10 min incubation, five phospho-PKC substrates (1, 2, 3, 4 and 5) were detected in the control (**Fig. 18I.c**), while two (1, 2) were detected in 20E treatment (**Fig.18I.d**). We located three protein spots on 2D gel (1, 2 and 3) (**Fig. 18I. a&b**) corresponding to Western blot signals on X-ray film (**Fig.18I. c&d**).

When SGs were treated with 1 μ M 20E for 30 min, seven phospho-PKC

substrates (1, 2, 7, 8, 9, 10 and 11) were detected by Western blot (**Fig. 18II.d**), compared with three (1, 2 and 6) detected in the controls (**Fig. 18II.c**), indicating that five additional PKC substrates (7, 8, 9, 10 and 11) were phosphorylated under 20E stimulation and two existing phosphorylated proteins (1, 2) were enhanced. Among the seven Western blot signals on the X-ray films, we located four protein spots (1, 2, 7 and 8) on the 2D gels (**Fig. 18II. a&b**). When SGs were treated for 60 min, two new PKC substrates (13, 14) were phosphorylated and one existing PKC substrate was enhanced (7) (**Fig. 18III. c&d**). At the same time, phosphorylation of protein # 7 was inhibited by 20E treatment. In this treatment, four protein spots (1, 2, 7 and 13) were located on 2D gel (**Fig. 18III. a&b**).



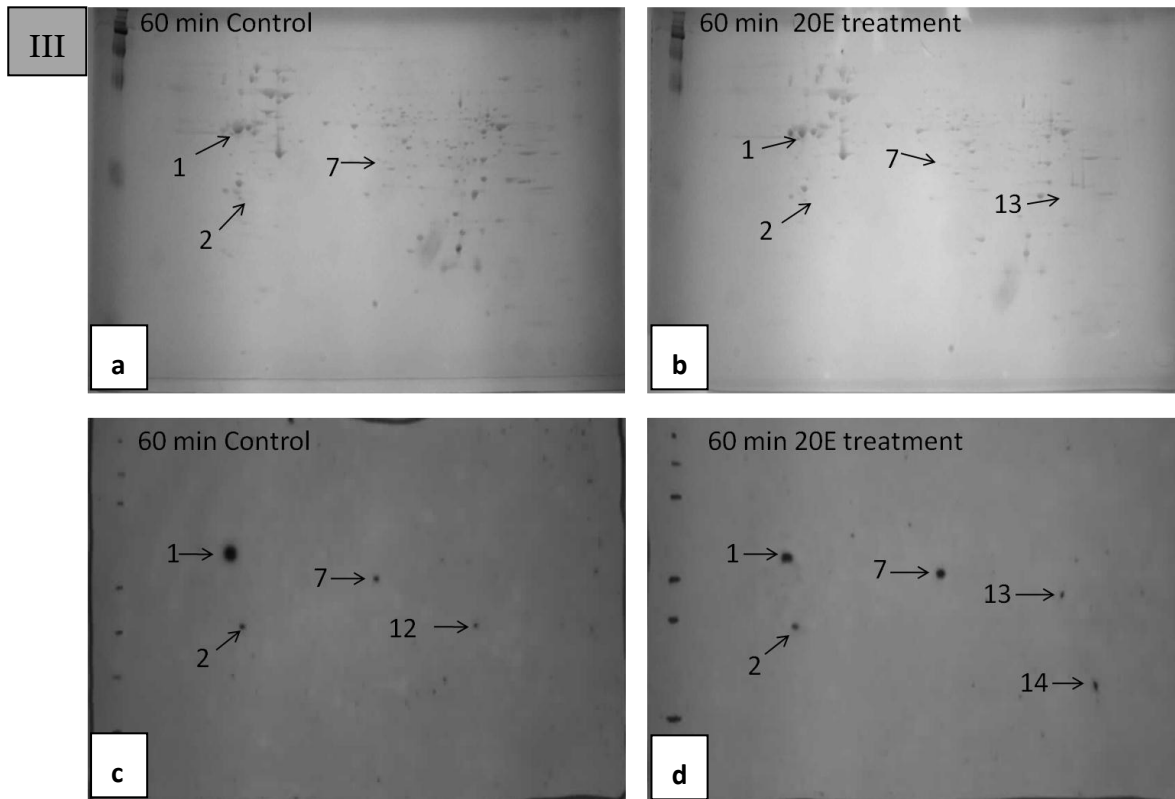


Fig. 18. 2D Western blot analysis of phospho-PKC substrates in the salivary glands. Seventy pairs of newly dissected salivary glands were incubated with or without $1\mu\text{M}$ 20E for 10 min (I), 30 min (II) and 60 min (III), then subjected to 2D gel separation and Western blot using a phospho-PKC substrate antibody. **a**, Coomassie blue stained 2D gels for control (no 20E); **b**, Coomassie blue stained 2D gels for 20E treatment; **c**, phosphorylated PKC substrate signals on X-ray films for control (no 20E); **d**, phosphorylated PKC substrate signals on X-ray films for 20E treatment.

The 2D Western blot analyses suggest that 20E stimulation could change the phosphorylation status of PKC substrates in SGs, by either inducing phosphorylation of new PKC substrates or inhibiting phosphorylation of existing phospho-PKC substrates. Furthermore, the 20E-regulated phosphorylation of

PKC substrates changed its profile over time, indicating a very complicated signal transduction pathway is involved. Unfortunately, we only located six protein spots (1, 2, 3, 7, 8 and 13) on 2D gels from the 14 Western blot signals, and were unable to find the corresponding protein spots for the other Western blot signals (4, 5, 6, 9, 10, 11, 12 and 14). This is very likely due to the lower sensitivity of Coomassie blue staining compared with Western blot.

MALDI-TOF MS/MS Identification

MS fingerprinting data were used to search for protein matches in the NCBI protein databank. Four of six proteins were identified in the gels (**Table 2**). As shown in Table 1, all four identified proteins had positive matches in a protein databank with MASCOT scores ranging from 93 to 548 (MASCOT score > 60 is significant, $p < 0.05$).

Protein Name	Spot number	Molecular Weight	Function	Score*
DHR23 (=RAD23)	1	45.8 kDa	damaged DNA repairing	548
eEF1delta, isoform B	2	28.9 kDa	translation elongation factor activity	391
lethal (1) G0334, isoform C	7	48.9kDa	pyruvate dehydrogenase	93
sterol carrier protein x	8	59 kDa	phospholipid transporter activity; sterol carrier activity; oxidoreductase activity.	197

Table 2. A list of phospho-PKC substrates identified in 2D Western blot analysis. MALDI-TOF MS/MS were used to generate peptide finger prints to search for matches against the NCBI nr database. * MASCOT scores, > 60 are significant ($p < 0.05$). Biological functions of identified proteins were searched at Uniprot.

3.4 Discussion

Although protein kinase C (PKC) family members are involved in a number of signal transduction processes including secretion, gene expression, proliferation and muscle contraction, many of their substrates remain unidentified. In vertebrates, PKC isozymes are subdivided into conventional PKCs (cPKC), novel PKCs (nPKC), atypical PKCs (aPKC) and PKC-related kinases (PKN). PKC α , β I, β II and γ isoforms belong to cPKC. When activated, cPKC isozymes phosphorylate substrates containing serine or threonine, with arginine or lysine

at the -3, -2 and +2 positions, and hydrophobic amino acids at position +1 (1-3).

The phospho-PKC substrate antibody from Cell Signaling Technology specifically recognizes this motif. As described on the instruction sheet, this antibody is suitable for all species, including insects. *M. sexta* is the first insect with successful application of this antibody (Rybczynski and Gilbert 2006). However, in *D. melanogaster*, no application has been reported yet. Our study provides the first evidence, showing that the antibody is applicable in the *Drosophila*.

As noted out in the previous chapters, 6 PKC isoforms have been found in *D. melanogaster*. Among them, PKC53E and eye-PKC belong to cPKCs. We speculate that the substrates identified by this Phospho-(Ser) PKC substrate antibody in SGs of *D. melanogaster* are substrates of PKC53E and eye-PKC. Since we have examined the PKC isoforms existing in SGs (Chapter 4), we already know that PKC53E, but not eye-PKC, exists in SGs of *D. melanogaster*, therefore, we infer PKC53E phosphorylated the PKC substrates detected in our experiments.

PKC undergoes a series of sequential phosphorylation steps to acquire full activation, including auto and trans-phosphorylation, which render them competent to interact with target proteins (Parekh et al. 2000; Newton 2003).

Our Western blot results showed that 20E-induced phosphorylation of new PKC substrates at 30 min and 60 min (**Fig. 18II** and **18III**), suggesting that 20E activates PKC activity. Although we do not know how 20E activates PKC in SGs, it is possible that Ca^{2+} and diacyl glycerol (DAG), the required factors of activation of cPKCs, are involved in this process. Recalling the recent discovery that ecdysteroid-response membrane receptor, a G-protein coupled receptor (GPCR) (Srivastava et al. 2005), transmits 20E signals through a phosphatidylinositol signal pathway, resulting in increased Ca^{2+} and DAG. We link the activation of PKC with 20E-induced nongenomic response through ecdysteroid-response membrane receptor. **Fig. 19** below depicts the relationship between GPCR and activation of PKC. However, this is a hypothesis to be further confirmed by experimental data.

We identified four PKC substrates from 2D western blot analysis. eEF1delta is one of the four subunits (alpha, beta, delta, and gamma) in the eukaryotic translation elongation factor 1 complex (EF-1). Phosphorylation of eEF1 beta, delta, and gamma units by PKC has been confirmed to stimulate the elongation

activity of the EF-1 complex up to three fold (Venema et al. 1991; Peters et al. 1995). 20E-induced molting process entails synthesis of a number of proteins, from which we infer that PKC-regulated phosphorylation of eEF1delta is necessary for 20E-induced protein expression.

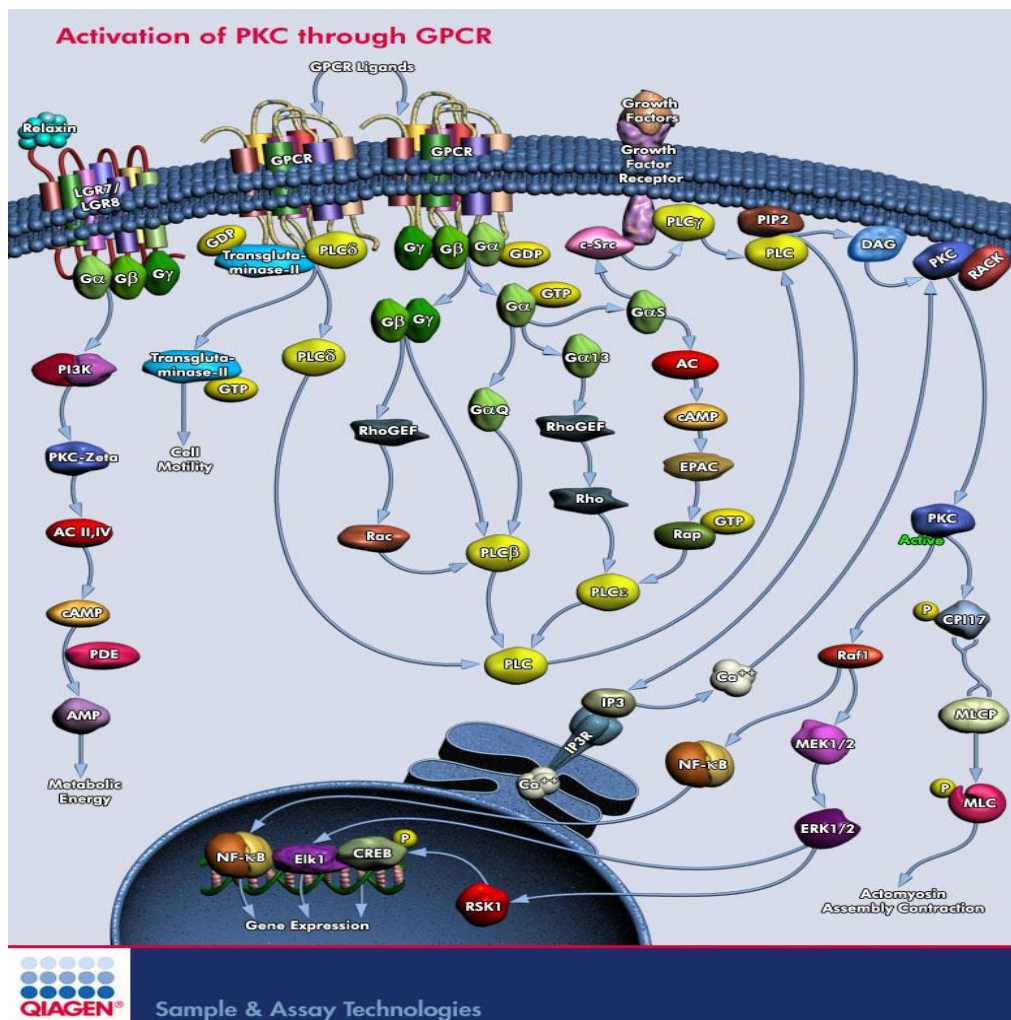


Fig. 19. Scheme of activation of PKC through hormone induced G protein-coupled receptor (GPCR) signal transduction.

(<https://www.qiagen.com/geneglobe/pathwayview.aspx?pathwayID=16>)

Sterol carrier protein X, a homologue of human steroid carrier protein-2 (SCP2), has a sterol binding domain and a thiolase domain. In humans, SCP2 has been studied as a nonspecific lipid-binding protein involved in the intracellular trafficking of cholesterol and phospholipid (Schroeder et al. 2007). SCP2 has been identified as a PKC substrate 20 years ago. The phosphorylation/dephosphorylation of SCP2 by PKC plays vital roles in the steroidogenesis regulated by tropic hormones (Steinschneider et al. 1989). Although we do not have evidence of 20E stimulating steroidogenesis, considering the sterol binding ability of SCP2, we hypothesize that phosphorylation of sterol carrier protein X may affect the transportation of 20E in the target cells.

For DHR23 and G0334 isoform C, so far, little is known about their phosphorylation. NetPhosK prediction results suggest that DHR23 and G0334 are PKC substrates (Score \geq 0.8) (**Table 3**).

PROTEIN NAME	SITE	KINASE	SCORE (THRESHOLD=0.8) (MAX=1.0)	NOTE
G0334	S-333	PKC	0.8	HIGHEST SCORE: 0.80 PKC AT POSITION 333
DHR23	S-79	PKC	0.8	HIGHEST SCORE: 0.90 PKC AT POSITION 342
	T-342	PKC	0.9	
STEROL CARRIER PROTEIN X	T-167	PKC	0.8	HIGHEST SCORE: 0.80 PKC AT POSITION 342
EEF1DELTA, ISOFORM B	T-109	PKC	0.77	HIGHEST SCORE: 0.77 PKC AT POSITION 109 (BELOW THE TRESHHOLD 0.8)

Table 3. Output of NetPhosK phosphorylation sites prediction.

(Method: NetPhosK without ESS filtering; Threshold=0.8).

DHR23, encoded by *Rad23*, is the homologue of vertebrate RAD23. RAD23 was originally identified as a factor involved in recognition of DNA lesions, also acts in targeting ubiquitylated proteins for proteasomal degradation and apoptosis (Brockstedt et al. 1998; Dantuma et al. 2009). Considering 20E-induced apoptosis in SGs, it is possible that PKC-regulated DHR23 phosphorylation is involved in the 20E-induced apoptosis process. We did not detect USP in our 2D Western blot analysis, likely because of the low amount of USP in total proteins.

Verification of PKC substrates

Verifications at protein level are necessary for phosphoproteomic analysis.

Although the bioinformatics software can provide some information about the identified proteins, we still need experimental data to verify that the proteins are PKC substrates and their phosphorylation is regulated by 20E. Western blot with antibodies against the identified proteins will be used to verify our phosphoproteomics results. Polyclonal antibodies against G0334, DHR23, sterol carrier protein X and eEF1delta isoform B will be elicited in rabbits with the recombinant proteins from bacteria culture expressing fragments of the above proteins. Two separate experiments are designed. First, we will verify that the identified proteins are PKC substrates. The expression of target proteins in SGs treated with PKC inhibitor and activator will be examined by Western blot with the synthesized antibodies. Then, the expression of target proteins in SGs under the challenge of 20E will be examined by Western blot as well, which enables us to confirm that the phosphorylation of identified proteins are stimulated by 20E.

Another approach to verify PKC substrates is *in vitro* kinase assay. The genes of G0334, DHR23, sterol carrier protein X and eEF1delta isoform B will be cloned into an expression vector which is optimized for the expression of Ser/Thr kinase substrates (e.g. pETPhos). The fusion proteins will be purified, and their phosphorylation will be examined in an *in vitro* kinase assay in the presence and absence of the recombinant PKC kinase domain and ATP. Phosphorylation of proteins will be monitored by immunodetection with phospho-(Ser) PKC substrate antibody (Linda Nova kova 2010). However, this approach can only confirm PKC substrates but cannot verify 20E-regulated phosphorylation.

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Appendix---Identification of PKC isoforms in the salivary gland of *Drosophila melanogaster* by RNA interference (Preliminary work and future work)

Introduction

PKCs are broadly conserved in eukaryotes, ranging in complexity from a single isoform in budding yeast (*Saccharomyces. cerevisiae*) to 6 isoforms in *D. melanogaster* and 12 in mammals (Mellor and Parker 1998; Shieh et al. 2002). In mammals, PKC isoforms play different roles in many cellular functions. For example, PKC θ (a novel PKC) is highly expressed by T cells and is involved in the activation of the transcription factor, nuclear factor- κ B (NF- κ B), nuclear factor of activated T cells (NFAT), and activator protein 1 (AP1); aPKCs (PKC ι and PKC ζ) in mammals mediate cell polarity; PKC β has been implicated in B cell activation, apoptosis induction, and endothelial cell proliferation (for review (Mackay and Twelves 2007; Rosse et al. 2010)).

In *D. melanogaster*, the six PKC isoforms: PKC 53E, PKC 98E, eye-PKC, atypical PKC (DaPKC), putative protein kinase C delta type homologue (PKC delta),

protein kinase related kinase N (CG2049), are involved in different biological processes. DaPKC acts in cell polarity formation through forming a complex with Baz (PAR-3) and PAR-6 (Ohno 2001; Tian and Deng 2008; Morais-de-Sa et al. 2010). The Baz/Par-6/aPKC complex also operates in polarizing the asymmetric divisions of *Drosophila* neuroblasts (Gonczy 2008; Siller and Doe 2009). Eye-PKC, an eye-specific PKC in photoreceptor cells, negatively regulates G-protein coupled PLC β signaling in photoreceptors. In the absence of eye-PKC, visual signals do not deactivate and the photoreceptor cells undergo light-dependent degeneration (Shieh et al. 2002). PKN, the protein kinase C-related kinase, is a Rho/Rac-binding kinase essential for *Drosophila* development and also needed in wing morphogenesis (Lu and Settleman 1999; Betson and Settleman 2007). As shown in Chapter 2, PKC activity is necessary for 20E signal transduction in SGs of *D. melanogaster*. Our question is: Which PKC isoform(s) is responsible for mediating 20E signal transduction in SGs of *D. melanogaster*?

To answer this question, we used RNA interference (RNAi) technology. RNAi has been used to discriminate protein isoforms in many works (Kisielow et al. 2002; Zhang et al. 2006; Chen et al. 2010). The rationale behind this experiment is that,

under ideal conditions, RNAi can knock down the expression of a PKC isoform through specifically designed double-strand RNA (dsRNA) with a sequence complementary to mRNA encoding a selected PKC isoform, while expression of other PKC isoforms remain unchanged. By examining the transcription of 20E-induced genes in PKC isoform knock-down salivary glands with quantitative real-time PCR, we determined which PKC isoform is necessary for 20E-induced gene expression. If a PKC isoform mediates 20E signal transduction, we expect to observe reduced transcriptions of 20E-induced genes.

RNAi has been successfully applied in *D. melanogaster* since 1998 (Kennerdell and Carthew 1998). The delivery of dsRNA to *D. melanogaster* has been performed both *in vitro* and *in vivo* ways. *In vivo*, microinjection and transgenic strain are two common ways to introduce RNAi molecules, either dsRNA or siRNA. For *in vitro*, the easiest system involves incubating the cells (from cell line) with the dsRNA added to the medium, as described for the first time using the *D. melanogaster* S2 cell line (Clemens et al. 2000). Since all of our previous experiments had been done in the SGs of *D. melanogaster*, we attempted to find a way to perform a SG-specific RNAi. Though we could generate transgenic

strains to carry out the RNAi assay, it could be very time consuming. Considering that the SG of *D. melanogaster* is a relatively simple tissue with small cell numbers and homogeneous cell composition (secretory cells and duct cells), and that the delivery of dsRNA by soaking works well in *Drosophila* cell lines (Clemens et al. 2000) and in the SG of the tick (Karim et al. 2004; Ramakrishnan et al. 2005), we decided to deliver dsRNAs to the SGs by soaking the SGs in a dsRNA- containing medium. Our results showed that it is a feasible way to perform RNAi in the SGs of *D. melanogaster* to investigate the function of specific PKC isoforms in the SGs.

Materials and Methods

PKC isoforms in the salivary gland

The full length cDNA sequences of *D. melanogaster* PKC isoforms, PKC 53E, PKC 98E, eye-PKC, DaPKC, PKC delta, CG2049, were acquired from NCBI by the accession numbers from Shieh's work in 2002 (Shieh et al. 2002). After sequence alignments (ClustalW 2.0), primers for each PKC isoform were designed. SGs from 3rd instar larvae, whole insects of 3rd instar larvae, and adults were

homogenized and RNAs were extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA concentration was determined by measuring the absorbance at 260 nm on a spectrophotometer. The extracted total RNA was treated with RNase-free DNase I (Promega, catalog# M6101) to eliminate the potential contamination by genomic DNA. About 1.5 µg of total RNA from each sample was used for the first-strand cDNA synthesis. The first-strand cDNA synthesis was primed using oligo (dT) based on the SuperScript First-Strand synthesis kit (Invitrogen, catalog# 11904-018). The synthesized cDNA was used as a template for estimation of gene transcription in SGs by PCR using the designed primers.

dsRNA production

The dsRNAs of PKC isoforms in the SGs were designed by **E-RNAi** (<http://www.dkfz.de/signaling2/e-rnai/>) with the length of dsRNA set between 350 and 450 bp. Two dsRNAs were selected for each PKC isoform and submitted to **dsCheck** (<http://dscheck.rnai.jp/>) for checking off-target effects of the designed sequences. A cDNA library derived from the SGs of 3rd instar larvae was used as a source of generating dsRNA templates. Then PCR with primers designed for specific dsRNAs was performed. For each dsRNA, we designed the

forward primer and backward primers containing a 5' T7 RNA polymerase binding site (TAATACGACTCACTATAGGG) followed by sequences specific for each dsRNAs (**Table 4**). PCR products were assessed by agarose gel electrophoresis and purified using a QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instruction. Then, dsRNAs were synthesized using an AmpliScribe™ T7 Transcription Reaction Kit following the manufacturer's instruction. GFP dsRNA was produced as a negative control. The reaction products were treated with RNase-free DNase I for 20 minutes incubation at 37°C to remove the DNA templates, and the dsRNAs were extracted with phenol/chloroform and precipitated with NaAc and ethanol. The quality of dsRNA was assessed by agarose gel electrophoresis and stored at -20°C until use.

PKC53E	Primer set 1 (449bp) Forward 5'- taatagactcactatagggcgattgggagaaaatcgaaa -3' Reverse 5'- taatagactcactataggggtaaggacgtgcctttgaa-3' Primer set 2 (428bp) Forward 5'- taatagactcactatagggccaagaagaagactcgacc-3' Reverse 5'- aatagactcactatagggctcagccagcaaacctttc-3
PKC98E	Primer set 1 (428bp) Forward: 5'-taatagactcactatagggatcatgaccctatgtctcc-3' Reverse: 5'-aatagactcactataggggaacttgtgccattcacct-3' Primer set 2 (419bp) Forward 5'-taatagactcactatagggggagaagcgcaacataaagc-3' Reverse 5'— taatagactcactataggggatcacgactacgactgga-3'
DaPKC	Primer set 1 (400bp) Forward 5'-taatagactcactataggggtgagtgccagtgtctta-3' Reverse 5'-taatagactcactataggggtgcaatctcgtagcaca-3' Primer set 2 (438bp)

	Forward 5'-taatacgactcactatagggtaaagaccgctacaatggc-3' Reverse 5'-taatacgactcactatagggactggatgcacttgaaaccc-3'
CG2049	Primer set 1(411bp) Forward 5'- taatacgactcactatagggtttgccgacaattaacca-3' Reverse 5'- taatacgactcactataggggccagtgagcaacaatcaaa-3' Primer set 2 (424bp) Forward 5'-taatacgactcactatagggggcttggtgtgtgatcctt-3' Reverse 5'- taatacgactcactatagggattcggccgtgatgaaaag-3'
GFP	Primer (385bp) Forward 5'-taatacgactcactatagggcacatgaagcagcagcactt-3' Reverse 5'- taatacgactcactatagggactgggtgctcaggtagtgg-3'

Table 4. Primers of PKC isoforms with T7 promoter.

RNA interference in the salivary gland

SGs were dissected from early wandering 3rd instar larvae and maintained in Grace's medium. Twenty pairs of SGs were transferred to Grace's medium with 0.5 µg/µl dsRNA of a different PKC isoform respectively and incubated at 37°C for 6h. As negative controls, twenty pairs of SGs were incubated with Grace's medium in the presence or absence of 0.5µg/µl GFP dsRNA. After 6 hours, the treated SGs were collected and total RNAs were extracted for RT-PCR. RT-PCR was performed to examine the expression of target PKC isoforms using PKC isoform specific primers.

Examination of 20E-induced genes with quantitative real-time PCR

SGs dissected from early wandering 3rd instar larvae were first treated with dsRNAs of PKC isoforms for 6 hours as previously described. After 6 hours, the medium was replaced with new Grace's medium containing 0.5 μ M 20E and SGs were incubated for another 6 hours at 37°C. After incubation, total RNA was extracted from treated salivary glands for cDNA synthesis. Quantitative real-time PCR (q-PCR) was performed to evaluate the transcription of three 20E-induced genes, including *E74A*, *E78B* and *DHR3*, using the Biosystems 7500 fast real-time PCR system (ABI). The final volume of reaction was 25 μ L using the ABI SYBR Green Supermix (ABI). For real-time PCR, the reaction was performed at 95°C for 10 min followed by 40 cycles at 95°C for 15 s, then 60°C for 1 min. The specificity of the SYBR Green PCR signal was confirmed by melting curve analysis and agarose gel electrophoresis. The mRNA expression was quantified using the comparative CT (cross threshold, the PCR cycle number that crosses the signal threshold) method (Livak and Schmittgen 2001). The CT of the housekeeping gene *rp49* was subtracted from CT of the target gene to obtain Δ CT. The normalized fold changes of the target gene mRNA expression were expressed as $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT$ is equal to $\Delta CT_{\text{treated sample}} - \Delta CT_{\text{control}}$.

Results

To identify the PKC isoforms existing in salivary glands, RT-PCR analysis was performed. As shown in **Fig. 19**, the bands corresponding to PKC53E, PKC98E, DaPKC and CG2049, but not eye-PKC and PKCdelta, were observed in the SG extract. For PKCdelta, it was detected in the whole body samples from both the larva and adult while Eye-PKC was detected only in the extract of adults, but not in the extract of larvae. RT-PCR results reveal that four PKC isoforms, i.e. PKC53E, PKC98E, DaPKC and CG2049, exist in the SGs of 3rd instar larva. Therefore, the four identified PKC isoforms were the targets for RNAi.

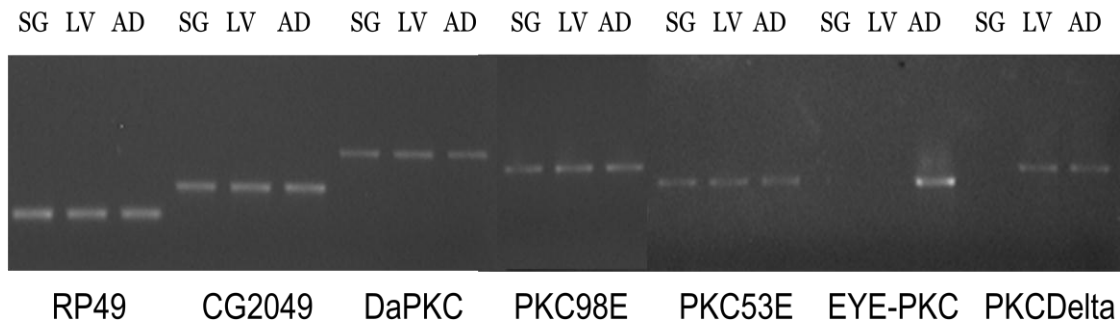


Fig. 19. RT-PCR analysis of PKC isoforms in the salivary glands of *D. melanogaster*. SG, salivary gland; LV, 3rd instar larva; AD, adult. *RP49* was used as a relative internal control.

Two dsRNAs for each of the four PKC isoforms in SGs were designed, then synthesized *in vitro* and analyzed by 1% agarose gel electrophoresis to ensure that the majority of the dsRNA existing as a single band at the appropriate sizes. The results showed that all synthesized dsRNAs appeared as a single, clear and bright band and their sizes were consistent with our design (**Fig. 20**). The concentrations of dsRNAs were measured by spectrometer at OD 260 and stored at -20°C until use.

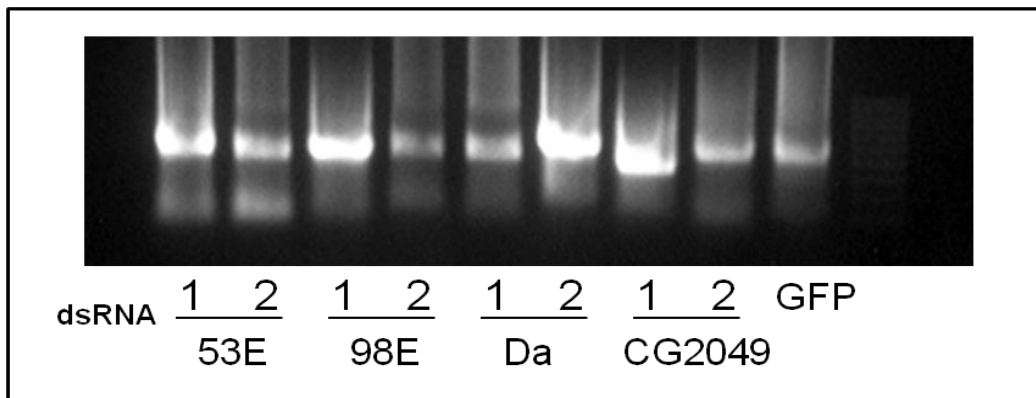


Fig. 20. Agarose gel electrophoretic analysis of synthesized dsRNAs of PKC isoforms. dsRNAs of the four PKC isoforms, PKC53E (**53E**), PKC98E (**98E**), DaPKC (**Da**), **CG2049** and a negative control-GFP were synthesized by *in vitro* transcription using the designed primers (**Table 4**). For each PKC isoform, two different dsRNA were synthesized (**1** and **2**).

The synthesized dsRNA 53E1, 98E1, Da1 and CG1 were used in the following RNAi experiments. By soaking SGs in medium with each of the synthesized dsRNAs, we introduced dsRNA to SGs and anticipated the knock-down of the selected PKC isoform. RT-PCR with isoform specific primers was used to verify the efficiency of RNAi. The results showed that three of the four PKC isoforms- PKC98E, CG2049 and PKC53E- showed obvious suppression of transcription after dsRNA treatment, while DaPKC mRNA did not show any detectable reduction with both Da1 and Da2 dsRNAs treatment (**Fig. 21**).

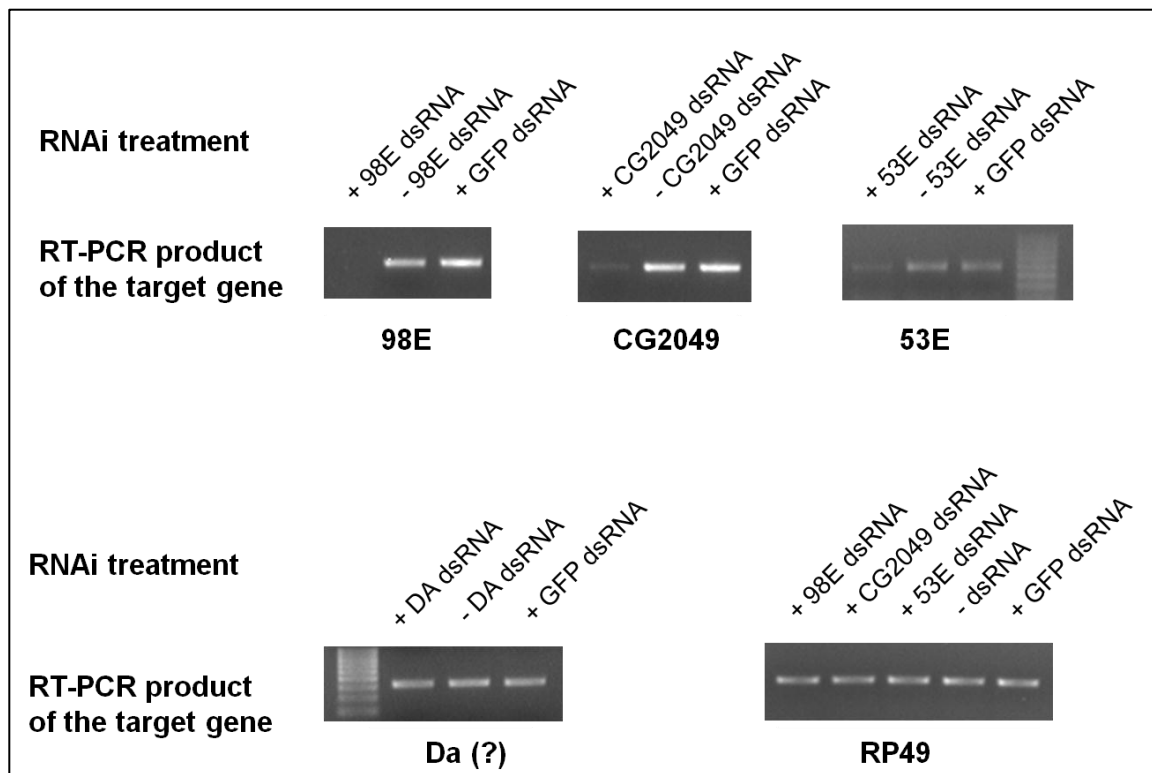


Fig. 21. RT-PCR analysis of PKC isoforms in salivary gland treated with dsRNAs. SGs treated with different PKC isoform dsRNAs were collected

and subjected to RT-PCR to analyze the transcription of PKC98E (**98E**), CG2049 (**CG2049**), PKC53E (**53E**), DaPKC (**Da**). RP49 was used as a relative internal control. dsRNA of GFP was used as negative control. 98E1, CG20491, 53E1 and Da1 dsRNA were used for dsRNA treatment. Da2 dsRNA was also used for dsRNA treatment and data are not shown here.

To determine which PKC isoform play roles in 20E signal transduction, we examined the transcript levels of three representative 20E-induced genes (*E78B*, *DHR3* and *E74A*) in PKC isoform knock-down SGs treated with 20E. However, real-time PCR analyses showed unexpected results (**Fig. 22**). In the PKC98E knock-down salivary glands, expressions of *E74A* were not very consistent, which showed a variation from 1 to 4 folds compared with the no dsRNA control; in CG2049 knock-down salivary glands, all test genes (*E78B*, *DHR3* and *E74A*) had higher transcript levels than that in the no dsRNA control. In contrast, expression of *E78B*, *DHR3* and *E74A* are completely suppressed in SGs with GFP dsRNA treatment, which was supposed to cause no change in this group.

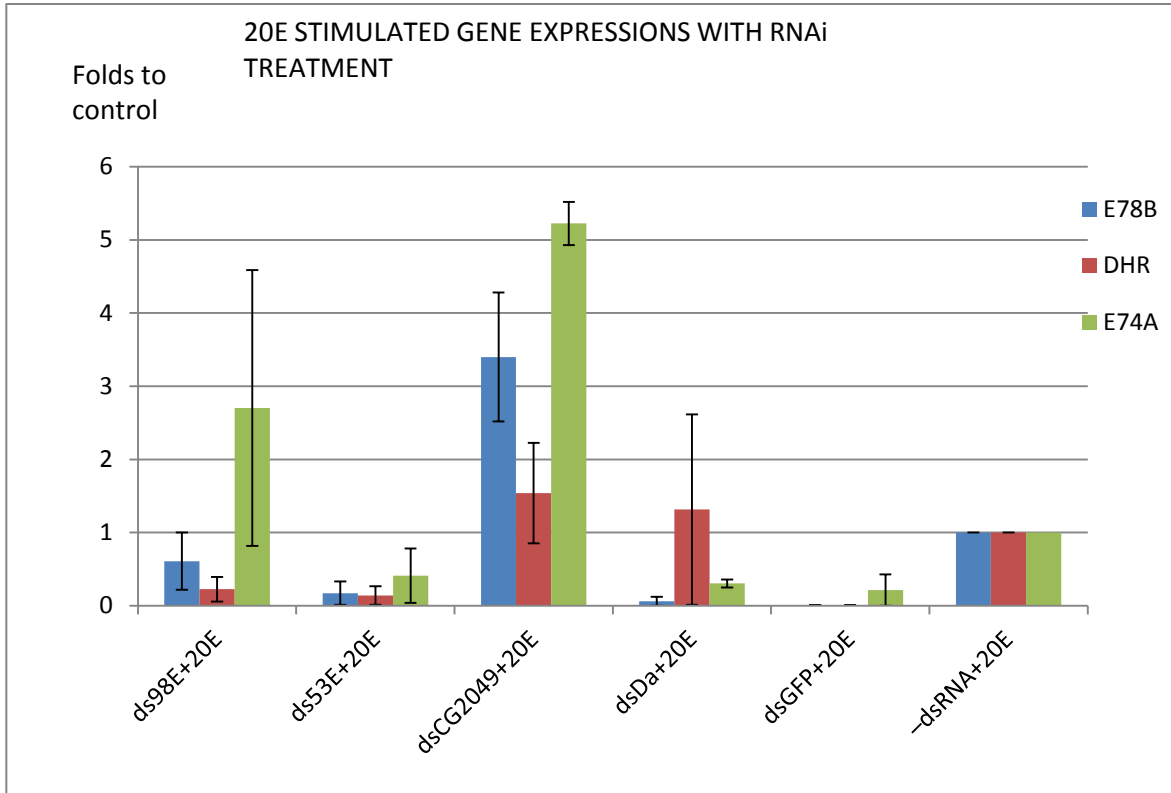


Fig. 22. Real-time PCR analysis of 20E-induced genes in PKC isoform knock-down salivary glands. Y axis, stands for the folds to control (-dsRNA+ 20E).

Discussion

We examined the expression profiles of the six *Drosophila* PKC isoforms in SGs of 3rd instar larvae, whole 3rd instar larvae and adults at the transcription level.

Four of six PKC isoforms were found in the SGs. Eye-PKC was only detectable in adults in our experiment, which is consistent with the in situ hybridization result in Shieh's paper (Shieh et al. 2002). For PKCdelta, it was detected in both larvae

and adults, but obviously, its expression is tissue specific and not in larval SG.

According to the data of *in situ* hybridization from Unigene EST profile

(<http://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer>) and gene report at

flybase (<http://flybase.org/>), PKC53E is only expressed in adult head, ; however,

we detected PKC53E in the SGs of larvae by RT-PCR, suggesting that PKC53E

may have additional function(s) besides phototransduction.

Though RNAi has been used in *D. melanogaster* for a long time and in many

different approaches, it is believed that *Drosophila* cells are unable to take up the

dsRNA in a systemic manner. Most RNAi research in *D. melanogaster* was done

by a transgenic approach (in cell expressed RNAi molecules) or cell line soaking

(large amount of RNAi molecules in the environment). Few reports of successful

RNAi application by soaking or feeding *D. melanogaster* with dsRNAs are

documented; only a few successful studies were reported in adults by dsRNA

injection (Dzitoyeva et al. 2001; Goto et al. 2003; Petruk et al. 2006). Here, we

showed for the first time the successful RNAi application by soaking the SGs of *D.*

melanogaster with dsRNAs. Three of four target genes (PKC isoform genes) were

evidently suppressed in SGs with dsRNA soaking treatment. A previous report in

D. melanogaster concluded that extracellular injection of dsRNA into *Drosophila* larvae cannot trigger RNAi in most *Drosophila* tissues, including the SG, due to the lack of the ability to uptake dsRNA from the surrounding environment (Miller et al. 2008). Although we did not use the exact same method to introduce dsRNAs, in our estimation, the small amount of dsRNA used in Miller's RNAi experiment (0.5 µg/larva) might be the reason for their unsuccessful RNAi. After injection, 0.5 µg dsRNA will be distributed to the whole body of an insect and for each tissue (e.g. SG), the environment around it will have a much lower dsRNA concentration compared with the concentration (0.5 µg/µl) used in our RNAi experiment.

To access the off-target effects of RNAi and to ensure that dsRNAs only suppress the selected PKC isoforms, we also examined the gene transcription of other PKC isoforms besides the target PKC isoforms (**Fig. 23**). The RT-PCR results showed that dsRNA of PKC53E and PKC98E strongly suppressed the transcription of their target PKC isoforms but has no effect on other isoforms. While, for dsRNA CG2049, besides its target isoform, CG2049, it also partially suppressed PKC98E and PKC53E, though not as strongly as CG2049. Thus, considering the

unsuccessful RNAi of DaPKC, we have two successful isoform-specific RNAi events (PKC53E and PKC98E), one partially successful event (CG2049) and one unsuccessful event (DaPKC). Our future work will be to redesign the dsRNA of DaPKC and CG2049 to suppress their own target genes and reduce off-target effects.

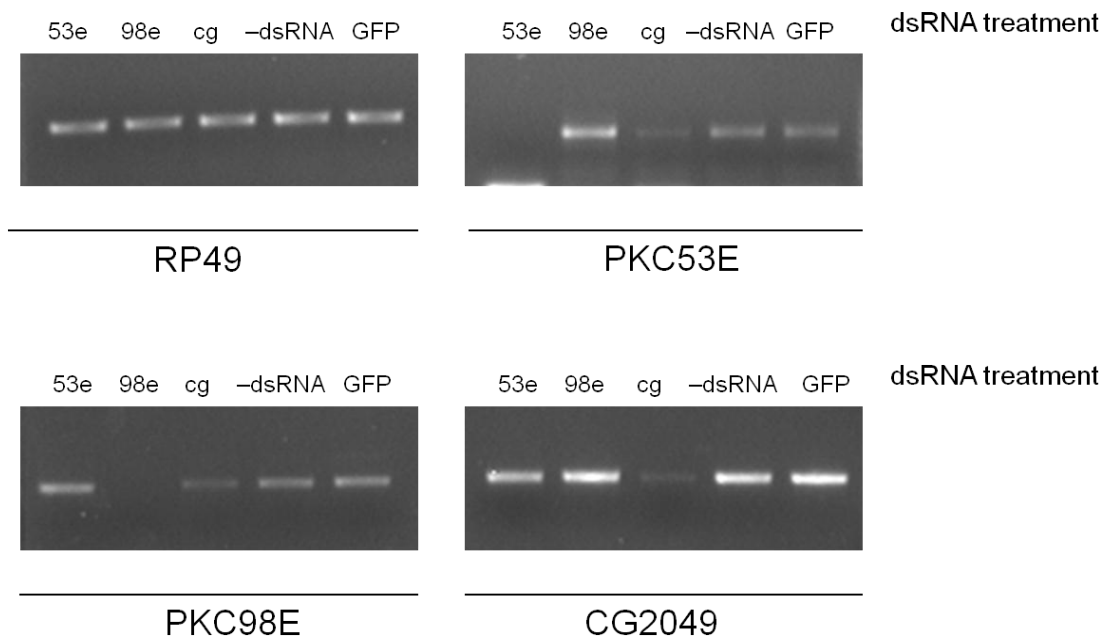


Fig. 23. RT-PCR analysis of RNAi off-target effect. RP49 was used as a relative internal control.

Though we have two successful RNAi events which successfully suppressed PKC53E and PKC98E respectively, we did not obtain any expected results from

the 20E-induced gene transcription. From the real-time PCR results, we had some RNAi events exhibit increased transcription of 20E-induced genes, yet some RNAi events, including dsRNA of GFP, totally suppressed transcription of 20E-induced genes. So, we think these real-time PCR data are not reliable and we need to repeat this assay in our subsequent research. Another consideration is 12 h (6h dsRNA soaking plus 6h 20E treatment) might not be long enough for RNAi and the turnover of PKC. According to research in yeast, the average degradation rate of 50 proteins is 2.2%/h with a range from imperceptible rates to 10%/h (Pratt et al. 2002). Therefore, after 12 hours of dsRNA treatment, there might be still a certain amount of PKC isoforms existing in salivary glands. We will extend the dsRNA soaking time in our next experiment, in an effort to obtain reproducible results.

To ensure the specificity of RNAi, we decided to add a positive control.

Myospheroid (*mys*), a gene encoding β PS integrin, is a good candidate for positive control. When RNAi this gene, it is known to cause changes in cell shape and cell adhesion in S2R+ cells (Kiger et al. 2003; Zhang and Hagen 2010).

According to the Flybase High Throughput Pattern Data (Beta version), *mys* is

highly expressed in the larval SG. Due to the big variation of GFP in real-time PCR in the preliminary experiment, we want to add another negative control in our RNAi experiment. LacZ is a good choice in terms of the successful application in the literature (Bai et al. 2008).

Future work

Although 20E-induced signal transduction pathway has been extensively studied in the last two decades, most of the studies were focused on the 20E-induced gene expression controlled by nuclear receptors (EcR and USP), namely genomic response; little is known about the fast response to 20E. Our data showed that 20E could stimulate PKC-regulated phosphorylation, suggesting that PKC is an intermediate in the 20E-induced fast response signal transduction pathway.

Based on our research of PKC-regulated phosphorylation in 20E signal transduction, we can further investigate either downstream or upstream of the PKC -involved 20E signal transduction. We have acquired some information about the downstream interaction of PKC through the phosphoproteomics work as described before; however, how PKC is activated still remains unclear.

Considering the discovery of an ecdysteroid membrane receptor, a GPCR, in

Drosophila (Srivastava et al. 2005), we speculate that 20E triggers the fast response through a membrane-located GPCR. Then PKC is activated through a pathway similar to Wnts-Frizzleds signal transduction pathway, in which PKC is activated by the increasing Ca^{2+} and DAG (Wang and Malbon 2003). To verify our speculation, we need to investigate the activation of some key proteins in the upstream of PKC activation in a GPCR activated PKC pathway, such as phospholipaseC beta ($\text{PLC}\beta$) and phosphoinositide 3-kinase ($\text{PI}3$). For the investigation of downstream of PKC activation, the identified PKC substrates in our experiment need to be verified by the methods described in chapter 3. The phosphorylation sites of the verified PKC substrates then can be studied by mass spectrometry analysis. Knowing the phosphorylation sites of a protein kinase substrate is very important to learn the function of the phosphorylation.

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VITA

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