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Transcriptional activation of the *Drosophila* ecdysone receptor by insect and plant ecdysteroids

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Abstract

A number of insect ecdysteroids, plant ecdysteroids and juvenoids were assayed for their ability to activate *Drosophila* nuclear receptors in transfected tissue culture cells. Discrete modifications to 20-hydroxyecdysone, the apparent natural ligand for the ecdysone receptor (EcR), conferred dramatic changes on the transcriptional activity of this receptor, suggesting that other biologically relevant EcR ligands may exist. Conversely, none of the compounds tested had a significant effect on the activity of three *Drosophila* orphan nuclear receptors: DHR38, DHR78 or DHR96. Taken together, these results demonstrate the selectivity of EcR for a series of natural and synthetic ecdysone agonists and suggest that as yet untested compounds may be responsible for activating DHR38, DHR78 and DHR96.

Keywords: Ecdysone receptor; Ultraspiracle; Orphan nuclear receptor; *Drosophila*; Ecdysteroids

1. Introduction

Pulses of ecdysteroids function as temporal cues that control the insect life cycle, triggering a wide range of developmental responses including molting and metamorphosis (Riddiford, 1993). Release of prothoracicotropic hormone (PTTH) from the central nervous system signals the synthesis and subsequent release of ecdysone from the ring gland (Henrich et al., 1987). This precursor is then modified by peripheral tissues into a wide range of ecdysteroids, including the biologically active hormone 20-hydroxyecdysone (20E) (Gilbert et al., 1996). 20E exerts its effects on development through a heterodimeric complex of two nuclear receptor superfamily members, ecdysone receptor (EcR) and Ultraspiracle (USP) which, in turn, trigger cascades of gene expression (for reviews, see Ashburner, 1974; Richards, 1997; Thummel, 1996). USP is the insect homolog of vertebrate RXR and, like its mammalian counterpart, functions as a heterodimer partner for the EcR ecdysone receptor, which determines the specificity of hormone recognition (Yao et al. 1992, 1993).

A second systemic hormonal signal has been identified in insects, juvenile hormone (JH), which acts to maintain the larval state until appropriate maturation has taken place (Riddiford, 1996). Although several lines of evidence suggest that JH acts through a nuclear receptor (Harmon et al., 1995; Jones and Sharp, 1997; Ismail et al., 1998), a specific JH receptor has not yet been identified.

In addition to the ecdysone receptor complex, a growing number of orphan nuclear receptors regulate *Drosophila* development, with at least half of these functioning in the 20E-regulated pathways of molting and metamorphosis (for a review, see Thummel, 1995). Three *Drosophila* orphan receptors, DHR38, DHR78 and DHR96, are expressed throughout development, but little is

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known about their transcriptional and mechanistic action (Fisk and Thummel, 1995). DHR38 is the insect homolog of the vertebrate NGFI-B nuclear receptor, and is required for proper development of the adult cuticle (Kozlova et al., 1998). Like its vertebrate homolog, DHR38 can heterodimerize with the RXR homolog USP, suggesting that it may define a unique hormone signaling pathway (Sutherland et al., 1995). Molecular and genetic studies of DHR78 have demonstrated that it can bind to a subset of target sites recognized by the EcR/USP heterodimer (Fisk and Thummel, 1995), and that it exerts an essential function during the third instar (Fisk and Thummel, 1998). DHR96 can also bind a subset of EcR/USP response elements, but its biological functions remain undefined (Fisk and Thummel, 1995).

A wide spectrum of ecdysteroids and juvenoids has been identified in the insect hemolymph, raising the possibility that at least some of these compounds may function as ligands for orphan nuclear receptors (Gilbert et al., 1996). In addition, given the specificity of nuclear receptors, it is important to establish a rank order of potency for ligands among receptors that function at the same place and time. The purpose of this study is to establish transcriptional response profiles for the ecdysone receptor using naturally occurring insect and plant ecdysteroids that have characteristics typical of nuclear receptor ligands (e.g., low molecular weight, lipophilic, etc.). Additionally, we have examined the effects of these and other candidate ligands on DHR38, DHR78 and DHR96, in an attempt to further elucidate their contribution to developmental signaling.

2. Materials and methods

2.1. Hormones

9-*Cis*-retinoic acid was purchased from Sigma. 22a-Hydroxycholesterol was purchased from Steraloids. 26-Hydroxyecdysone was isolated from eggs (0–12 h old) as described previously (Warren et al., 1986), while 3-dehydroecdysteroids were chemically oxidized from the corresponding parent compounds. 3-(α)-Epiecdysteroids were synthesized by chemical reduction of the corresponding 3-dehydroecdysteroids and purified from the by-products, i.e., from 20E and makisterone A (Spindler et al., 1977; Dinan and Rees, 1978). Inokosterone, ponasterone and 22-isoecdysone were gifts of Professor Koji Nakanishi, while 2-deoxyecdysone, 7-dehydro-25-hydroxycholesterol and 7-dehydro-25-hydroxycholesterol-5,8-peroxide were gifts of Professor René Lafont. α-5,6-Epoxy-7-dehydrocholesterol, α-5,6-epoxycholesterol and β-5,6-epoxycholesterol were synthesized as described previously (Warren et al., 1995). RH5489 and RH5992 were a gift of the Rohm and Haas Company. All other ecdysteroids were obtained from commercial suppliers. Stock solutions of compounds were dissolved in 1:1 solution of ethanol/dimethyl sulfoxide (DMSO).

2.2. Cell culture

SL2 Schneider cells, an embryonic *Drosophila* cell line, were grown in Schneider's Drosophila medium (GIBCO) supplemented with 13.5% super-stripped fetal bovine serum (Gemini) and 1% antibiotic–antimycotic (GIBCO) in atmospheric conditions at 24°C.

2.3. Plasmids

pA5C–EcR was a gift from Dr William A. Segraves. Chimeric Gal4–orphan receptor expression plasmids were created by fusing the yeast Gal4 DNA binding domain (DBD) to the ligand binding domain (LBD) of the respective receptor using the *Drosophila* pA5C expression vector (Koelle et al., 1991). pA5C–GalDHR38 was constructed as follows: the LBD of DHR38 was amplified by polymerase chain reaction (PCR) using pBluescript–DHR38 as a template (Fisk and Thummel, 1995) and T7 and DHR38.GALLBD–RI (5′ CCTGGTCGTAGAATTCGTCAAGGAAGTG 3′) primers. The DHR38.GALLBD–RI primer contains an Eco RI site. The resulting PCR product was digested with Eco RI and Eco RV, and inserted into pCMX–Gal4 (Willy et al., 1995). This construct encodes a protein with the Gal4 DBD fused in-frame to the DHR38 LBD beginning at amino acid 288. GalDHR38 was then excised from the pCMX vector by digestion with Hind III and Nhe I, blunted with Klenow DNA polymerase and inserted into the Eco RV site of pA5C. pA5C–GalDHR78 was constructed as follows: the LBD of DHR78 was amplified by PCR using pBluescript–DHR78 as a template (Fisk and Thummel, 1995) and DHR78.GALLBD–RI (5′ AAAGAATTCCGAAGT GATTCTGTGCAC 3′) and DHR78.REV–BAM (5′ AAAGGATCCGCCTACAGTCCACTAGTGTTG 3′) primers. The resulting PCR product was digested with Eco RI and Bam HI enzymes, and inserted into pCMX–Gal4. The plasmid encodes a protein that contains the Gal4 DBD fused in-frame to the LBD of DHR78 at amino acid 118. GalDHR78 was excised from pCMX with Hind III (blunted) and Bam HI, and inserted into pA5C between the Eco RV and Bam HI sites. PACKN–GalDHR96 was constructed as follows: the LBD of DHR96 was amplified by PCR using pBluescript–DHR96 as template (Fisk and Thummel, 1995) and DHR96.GALLBD–BAM (5′ AACGGGATCCAGA GTTGAAAACATTATGTCC 3′) and DHR96.REV–NHE (5′ AAAGCTAGCATCGGTTGTCTAGTGATT TTTC 3′) primers. The resulting PCR product was digested with Bam HI and Nhe I, and inserted into pCMX–Gal4. The plasmid encodes a protein that contains the Gal4 DBD fused in-frame to the LBD of

DHR96 at amino acid 74. GalDHR96 was excised from pCMX with Hind III (blunted) and Nhe I, and inserted into the PACKN vector between the Xho I (blunted) and Nhe I sites.

The ecdysone response reporter plasmids pADH–hspEcRE–LUC and pADH–UAS–LUC were derived from the pAdh vector (Koelle et al., 1991). These plasmids contain either the *hsp27* ecdysone responsive element (EcRE) or the yeast Gal4 upstream activating sequence (UAS) inserted just upstream of the minimal *Drosophila Adh* promoter, which drives expression of the firefly luciferase gene. The following oligonucleotides were synthesized for generation of pADH–hspEcRE–LUC and pADH–UAS–LUC: 5′ AGCTTCA GGTCATTGACCTGAG 3′, 5′ AGCTCTCAGGTC AATGACCTGA 3′, 5′ AGCTCGGAGTACTG TCCTCCG 3′ and 5′ AGCTCGGAGGACAG TACTCCG 3′. Three tandem copies of these oligonucleotides, which have compatible Hind III overhangs, were inserted at the Hind III site of pADH.

2.4. Transfection

100 μl/well of SL2 cells at a density of 8×10^5 cells/ml were plated in 96-well opaque plates (Costar), covered with Parafilm, and allowed to grow overnight. After ~17 h of growth, 20 μl of transfection mix was added per well. Transfection mix was prepared using the standard calcium phosphate method buffered with 1X 4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid (HEPES) pH 7.4 and contained 25 ng receptor, 15 ng reporter, 5 ng internal control plasmid (A5C–βGal, which drives high-level expression of the *E. coli* β-galactosidase protein) and 100 ng carrier DNA (pGEM) for each well that was transfected. Cells were dosed with 20 μl of the indicated compounds in media 6 h post-transfection and harvested 17 h after dosing with hormone. The medium in each well was replaced by 50 μl per well of luciferase lysis buffer [3 mM tricine pH 7.8, 0.8 mM magnesium acetate, 0.02 mM ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid (EDTA), 0.15 mM adenosine triphosphate (ATP), 100 mM 2-mercaptoethanol, 1% Triton X100, 0.5 mM Coenzyme A (Sigma) and 0.5 mM D-luciferin, sodium salt (Molecular Probes)] and the plates were incubated at room temperature under aluminum foil for 30 s. Light units were then read with an AML-34 luminometer (Torcon). Cell lysates were transferred to a clear 96-well plate (Costar) and 100 μl/well of 2-Nitrophenyl-β-D-galactopyranoside (ONPG) buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4) containing 2 mg/ml ONPG was added. After color development at 37°C, the reaction was terminated with the addition of 50 μl/well 1 M sodium carbonate. β-Galactosidase activity was measured on a Dynatech MR5000 plate reader (test filter 410 nm, reference filter 630 nm). The relative light units (RLU) reported in Figs. 1 and 2 were calculated as: (light units/OD 420)×reaction time.

3. Results and discussion

Although the identification of the EcR/USP heterodimer as the functional ecdysone receptor is well documented (Koelle, 1992; Yao et al., 1993), the structure/activity relationship of ecdysone receptor agonists has not yet been fully characterized. To better understand the ligand selectivity of the EcR/USP heterodimer, a directed series of insect and plant ecdysteroids was assayed for their ability to induce ecdysone-receptor-mediated transcription of a luciferase reporter gene in transfected tissue culture cells. This cotransfection assay has been widely used to characterize ligands for many vertebrate nuclear receptors; in addition, this assay has been crucial to the identification of novel ligands for vertebrate orphan receptors (for examples, see Kliewer et al., 1998; Janowski et al., 1996; Janowski et al., 1999; Makishima et al., 1999). To implement our studies, a reporter gene (pADH–hspEcRE–LUC) was cotransfected with an EcR expression plasmid (pA5C–EcR) into *Drosophila* SL2 cells, which express significant quantities of endogenous USP (Yao et al., 1992). The reporter plasmid carries three tandem copies of the *hsp27* ecdysone response element (EcRE), which functions as a strong binding site for the EcR/USP heterodimer (Yao et al., 1993). Insect ecdysteroids with modifications at the C3, C20 or C24 position [Fig. 1(A) and (B)] all had varying effects on ecdysone-receptor-mediated activation of the luciferase reporter gene [Fig. 1(C) and (D), Table 1].

Of the insect ecdysteroids, 20E was the most potent and among the most efficacious (EC_{50}=0.18 mM), followed by makisterone A (EC_{50}=0.29 mM) (Fig. 1, Table 1). In contrast to previous reports that used insect bioassays to monitor activity (Weirich et al., 1989; Sommé-Martin et al., 1990; Bergamasco and Horn, 1980; Nigg et al., 1974; Kaplanis et al., 1979), we found that 3-epi-20-hydroxyecdysone has a high efficacy, although it is a relatively poor activator at lower concentrations [Fig. 1(D), Table 1]. This resulted in an EC_{50} for 3-epi-20-hydroxyecdysone (4.2 mM) that is an order of magnitude greater than the EC_{50} obtained for either 20E or makisterone A [Fig. 1(C), Table 1]. Further modifications to 20E or makisterone A markedly reduced activity. In addition, ecdysone, which is secreted from the ring gland and primarily converted to 20E in the fat body by the cytochrome P450 enzyme ecdysone-20-monooxygenase (Smith and Mitchell, 1986), had only modest effects on transcription, even at the highest concentrations tested (10 mM), and was similar to 3-epimakisterone [Fig. 1(D)]. Interestingly, 3-dehydro-20-hydroxyecdysone, a downstream metabolite of 20E (Grau and Lafont, 1994;

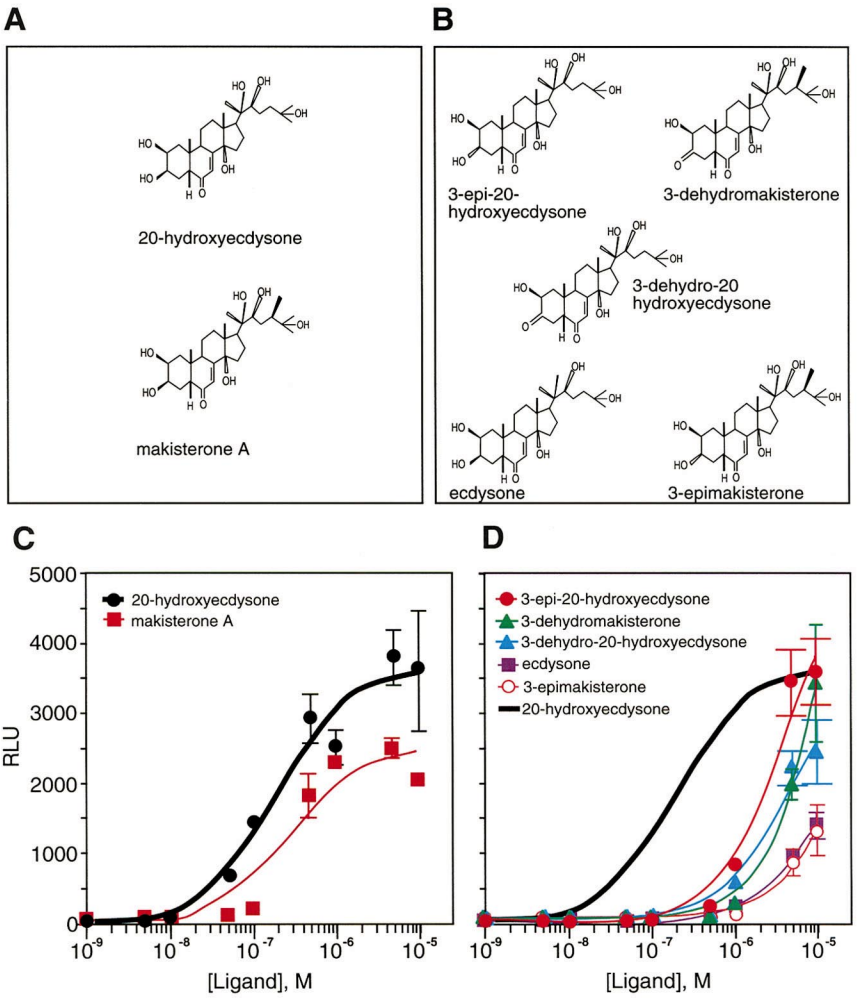

Fig. 1. Dose–response curves for activation of EcR by insect ecdysteroids. Shown are the structures and dose–response curves of *Drosophila* ecdysteroids that have high (A and C) or low (B and D) potency for activating EcR. The dose–response curve for 20-hydroxyecdysone generated in (C) is provided in (D) as a bold solid line for comparative purposes. Compounds were assayed for their ability to activate transcription of an ecdysone-inducible luciferase reporter gene in SL2 cells. Transcriptional activation is represented as relative light units (RLU), and is plotted relative to the concentration of the compound tested. Individual points in (C) and (D) are the average±standard deviation from three independent experiments.

Sommé-Martin et al., 1988; Cherbas and Cherbas, 1970), has been shown to have a potency indistinguishable from 20E in *Manduca* (Hiruma et al., 1997) and was also observed to have high activity in *Drosophila* larval fat body (Sommé-Martin et al., 1990), but is a relatively poor activator of EcR/USP when tested in the *Drosophila* cotransfection assay [Fig. 1(D)].

Dose–response curves for the *Drosophila* ecdysone receptor were also generated with phytoecdysteroids (Fig. 2). 20E deoxygenated at the C25 position [ponasterone A; see Fig. 2(A)] proved to be the most potent activator of EcR among all compounds tested. Ponasterone A was clearly distinguishable from 20E, having a lower EC_{50} (significant activation was observed with a concentration as low as 1 nM) and a higher efficacy [Fig. 2(C), Table 1]. This finding is consistent with a previous report by Cherbas et al. (1980) which showed that ponasterone A is eight times more effective than 20E at initiating the *Drosophila* Kc-H cell morphological response. Hydroxylation at the C5 and C11 positions yields muristerone A [Fig. 2(A)], another phytoecdysteroid with potent ecdysteroid activity (Yao et al., 1993). Consistent with previous reports (Yao et al., 1993), muristerone A initiated the highest levels of EcR/USP-mediated transcription in the cotransfection assay, and had an EC_{50} comparable to that of the natural ligand 20E [Fig. 2(C), Table 1]. Although toxic at high concentrations, cyasterone, which contains a large side-chain modification at the C24 position [Fig. 2(B)], was also a potent EcR agonist [Fig. 2(D)]. Relatively poor plant ligands for EcR were inokosterone and 2-deoxy-20-hydroxyecdysone [Fig. 2(B) and (D)]. Cyasterone, inokosterone and 2-deoxy-20-hydroxyecdysone were previously shown to have relative activities of 0.5, 0.11 and 0.03, respectively, when compared with 20E in the Kc-H cell assay (Cherbas et al., 1980). Finally, the non-

Table 1
Compounds that activate[a] the ecdysone receptor

Compound	EC_{50} [b] (μm)	Efficacy[c] (%)
20-Hydroxyecdysone[d]	0.18	100
Ponasterone A	0.07	124.6
Makisterone A[d]	0.29	56.5
Cyasterone	0.33	[e]
Inokosterone	1.4	69.1
Muristerone A	1.8	175
3-Epi-20-hydroxyecdysone[d]	4.2	103.2
3-Dehydro-20-hydroxyecdysone[d]	4.7	69.4
3-Dehydromakisterone[d]	d.n.s.[f]	93.5
Ecdysone[d]	d.n.s.	38.8
3-Epimakisterone[d]	d.n.s.	35.6
RH5992	d.n.s.	28.5
2-Deoxy-20-hydroxyecdysone	d.n.s.	20.2

[a] Compounds tested but not active were: 26-hydroxyecdysone; 3-dehydroecdysone; 22-isoecdysone; 14,22,25-trideoxyecdysone; 22,25-dideoxy-5α-(H)-ecdysone; 5β-(H)-cholestane-6-one-2β,3β-diol; 22α-hydroxycholesterol; 5α,6α-iminocholesterol; 5β,6β-iminocholesterol; 5β,6β-iminocholesterol-*N*-acetate; 5α,6α-epoxycholesterol; 5β,6β-epoxycholesterol; 5α,6α-epoxy-7-dehydrocholesterol; 5α,6α-epoxy-7-dehydrocholesterol-N_3-OH adduct; 7-dehydro-25-hydroxycholesterol; 7-dehydro-5α,8α-peroxide-25-hydroxycholesterol; 7α-hydroxycholesterol; 7β-hydroxycholesterol; 5-cholestene-3β-ol-7-one; 5α-choles-7-ene-3β-ol; cholesta-4-ene-3-one; cholesta-5-ene-3-one; 5-pregnene-3β,20β-diol; 5-pregnene-3β,20α-diol; RH5849; 9-*cis*-retinoic acid; JH0; JHI; JHII; JHIII; JHIII bisepoxide; JH acid; hyrdoprene; methoprene; fenoxycarb; farnesoic acid; and methoprene acid.

[b] EC_{50}=effective concentration for 50% maximal activation.

[c] Efficacy=maximal activation at 10 μM relative to 20-hydroxyecdysone.

[d] Naturally occurring *Drosophila* ecdysteroid.

[e] Toxic at 10 μM.

[f] d.n.s.=does not saturate.

steroidal compound RH5992 [Fig. 2(B)], which has been reported to have an ecdysone-receptor-dependent LC_{50} (lethal dose concentration where 50% of cells die) of 600 nM in C18+ *Drosophila* imaginal disk cells (Cottam and Milner, 1997), had only modest affects on EcR/USP-mediated transcription [Fig. 2(D), Table 1]. It should be noted that, although SL2 cells were used in the experiments reported here, no significant metabolism of 20E has been seen in Kc cells, a related *Drosophila* cell line (L. Cherbas and P. Cherbas, personal communication).

Table 1 lists 37 other compounds (ecdysteroids, cholesterol derivatives, pregnanes, a retinoid and juvenoids) that were tested in the ecdysone receptor transcriptional assay, all of which had no significant activity (data not shown). Together with the EcR agonists, these compounds were also tested for their ability to activate Gal4 chimeras (Harmon et al., 1995) in which the Gal4 DNA binding domain is fused to the ligand binding domains of the DHR38, DHR78 and DHR96 orphan receptors. However, in each case, the measured level of transcriptional activity for these receptors did not exceed those that were treated with vehicle alone (data not shown). The observation that natural and synthetic juvenoids failed to activate the EcR/USP heterodimer and the orphan receptors tested suggests that USP is not a juvenile hormone receptor in the context of a homodimer or a heterodimer with EcR or DHR38. This is despite the fact that USP is the insect homolog to the mammalian receptor RXR, and RXR can be activated by retinoids and juvenile hormone analogues (Heyman et al., 1992; Harmon et al., 1995), compounds that have isoprenyl chains similar to those found in juvenoids. The possibility still remains, however, that USP may serve a role in juvenoid-mediated signaling when paired with an unidentified partner.

This report represents the first comprehensive study of the effects of insect and plant ecdysteroids on the transcriptional activity of the EcR/USP heterodimer in *Drosophila* cells. Our transcriptional data correspond relatively well with a previous study by Mao et al. (1995). This group defined the binding affinities of ecdysteroids in the ixodid tick and found the relative affinities to be: ponasterone A>muristerone A>makisterone A>20E>ecdysone. Our data are also in agreement with Hiruma et al. (1997), who showed in vivo activity for the induction of mRNA in *Manduca sexta* for 20E and 3-dehydro-20-hydroxyecdysone, but failed to see biological activity for 3-dehydroecdysone and 26-hydroxyecdysone. As previously noted, a study performed by Cherbas et al. (1980) detailed the effectiveness of different ecdysteroids in the extension of processes in *Drosophila* Kc-H cells. Again, their qualitative assignments of cell morphology correspond well with the quantitative measurements obtained in this report for the induction of an ecdysone-receptor-inducible reporter.

The observation that the natural ecdysteroid makisterone A is able to generate a half-maximal transcriptional response in a range approaching that of 20E raises some interesting questions regarding the true in vivo ligand for the ecdysone receptor in *Drosophila*. Indeed, makisterone A may play a role in ecdysone receptor signaling throughout development. The titers of individual ecdysteroids supplied by the ring gland change drastically and, as demonstrated by Pak and Gilbert (1987), the most abundant free ecdysteroids at pupariation in *Drosophila* are makisterone A and 20E. Redfern (1986) noted that an increase in dietary campesterol resulted in the increase of 20-deoxymakisterone A relative to ecdysone, which are converted to makisterone A and 20E, respectively. That makisterone A may serve the role of 20E in insect signaling is not unprecedented. For example, makisterone A is thought to be the major ecdysteroid in the last larval instar of the honeybee (Rachinsky et al., 1990).

Continued effort in the area of ecdysteroid and lipid research in insects should provide solutions to unanswered questions in insect nuclear receptor signaling. Identification of novel ligands for the insect orphan receptors, as well as the mechanisms of action behind

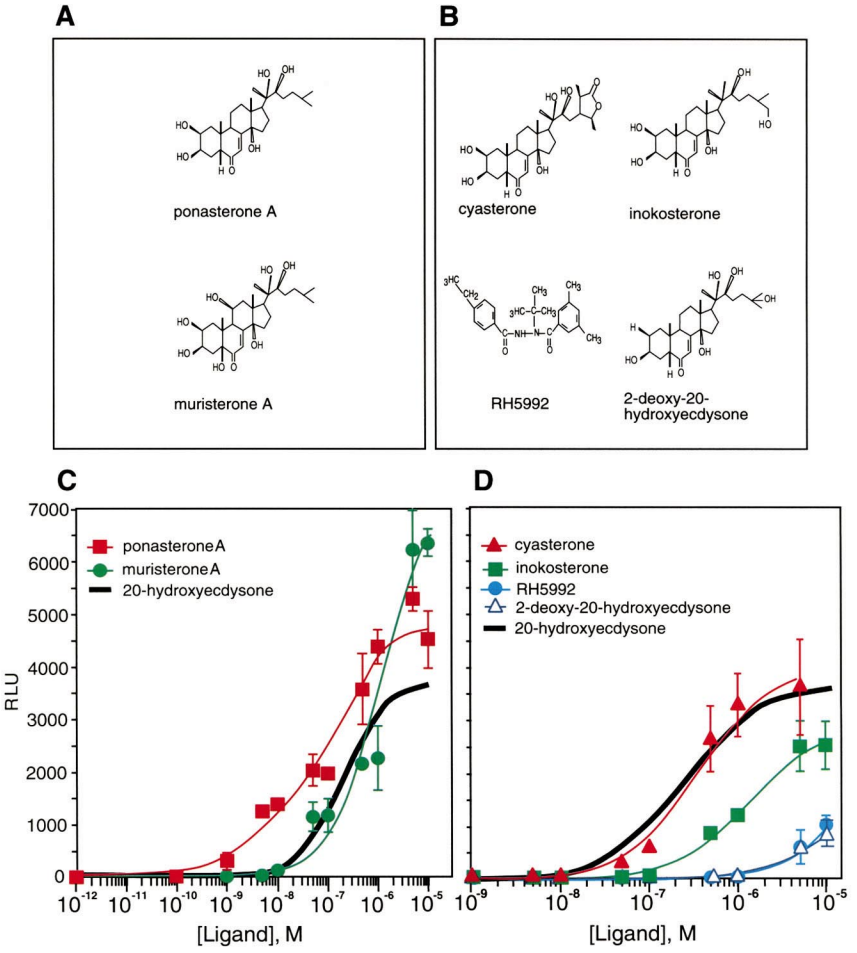

Fig. 2. Dose–response curves for activation of EcR by phytoecdysteroids and a synthetic analog. Shown are the structures and dose–response curves of phytoecdysteroids that have high potency (A and C) or phytoecdysteroids and a synthetic analog that have low potency (B and D) for activating EcR. The highest concentration of cyasterone in (D) is toxic and is therefore not shown. The dose–response curve for 20-hydroxyecdysone generated in Fig. 1(C) is provided as a solid bold line in (C) and (D) for comparative purposes. Compounds were assayed for their ability to activate transcription of an ecdysone-inducible luciferase reporter gene in SL2 cells, as in Fig. 1. Transcriptional activation is represented as relative light units (RLU), and is plotted relative to the concentration of the compound tested. Individual points in (C) and (D) are the average±standard deviation from three independent experiments.

transcriptional activation, provide important future directions for understanding the hormonal regulation of insect development.

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