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REGULATION OF CYTOCHROME P-450 DEPENDENT STEROID HYDROXYLASE ACTIVITY IN *MANDUCA SEXTA*: EFFECTS OF THE ECDYSONE AGONIST RH 5849 ON ECDYSONE 20-MONOOXYGENASE ACTIVITY

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SUMMARY. The non-steroidal ecdysone agonist RH 5849 (1,2-dibenzoyl-1-*tert*-butylhydrazine) was found to inhibit in a dose-response and apparently competitive fashion the cytochrome P-450 dependent ecdysone 20-monooxygenase activity in the midgut of wandering stage last instar larvae of the tobacco hornworm, *Manduca sexta*. More effectively on a per molar basis than the naturally occurring molting hormones ecdysone and 20-hydroxyecdysone, RH 5849 was also found to elicit the dramatic 50-fold increase in midgut steroid hydroxylase activity (which normally occurs with the onset of the wandering stage) when injected into competent head or thoracic ligated pre-wandering last instar larvae. These data support and extend the potential usefulness of RH 5849 as a pharmacological probe for further investigating the actions of ecdysteroids and their role(s) in the regulation of ecdysteroid monooxygenases.

INTRODUCTION. Ecdysone 20-monooxygenase (EC 1.14.99.22) is the insect steroid hydroxylase system responsible for the conversion of the molting hormone ecdysone (2β,3β,14α,22R,25-pentahydroxy-5β-cholest-7-en-6-one) to its more physiologically active metabolite 20(R)-hydroxyecdysone (1). In addition to the intrinsic importance of the reaction catalyzed by this enzyme, ecdysone 20-monooxygenase has been studied extensively as a model for understanding the prothoracic gland steroid hydroxylases responsible in large part for the synthesis of ecdysone from cholesterol or plant sterols (1-3).

Several studies have revealed that ecdysone 20-monooxygenase is essentially an NADPH requiring cytochrome P-450 dependent steroid hydroxylase system similar in properties to vertebrate enzymes such as the cholesterol side chain cleavage and 11β-hydroxylase systems (4-9). *In vivo* studies have suggested and *in vitro* measurements have confirmed that ecdysone 20-monooxygenase activity fluctuates dramatically and in a tissue specific fashion during the insect life cycle (10-17). The dramatic stage and tissue specific fluctuations in this enzyme activity are in accord with the view that this steroid hydroxylase system is itself under some form of regulation. Previous studies have implicated ecdysone and 20-hydroxyecdysone as the principal regulators of ecdysone 20-monooxygenase activity (17-20). Accordingly, in the present study we examined the

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effects of the recently discovered non-steroidal ecdysone agonist RH 5849 (21, 22) on ecdysone 20-monooxygenase activity in the midgut of the tobacco hornworm, *Manduca sexta*, during larval-pupal development.

MATERIALS AND METHODS. Animals. The animals used in this investigation were last instar larvae of the tobacco hornworm, *Manduca sexta*. Animals were reared on an artificial diet under a non-diapausing photoperiod (L:D, 16:8) at 26°C and 60% relative humidity (23). Animals were staged as previously described (24) and only gate II fifth instar larvae (days 3 to 5 of the stadium) were used in these studies.

Ecdysteroids and Chemicals. The radiolabelled ecdysteroid substrate for the monooxygenase assay was [23,24-^{3}H]-ecdysone (stocks of 45 and 70 Ci/mmol) purchased from New England Nuclear, Boston, MA. Ecdysteroid standards and NADPH were purchased from Fluka Chemical Corp., Ronkonkoma, NY, and Sigma Chemical Co., St. Louis, MO, respectively; salts, organic solvents, and scintillation fluid (ScintiVerse E) were purchased from Fisher Scientific Co., Cleveland, OH; RH 5849 was a gift from Dr. Keith Wing, Rohm and Haas Company, Spring House, PA.

Animal Ligations and Injections. Animals to be ligated and injected were chilled on ice. Head (between head and prothoracic segment) and thoracic (between metathorax and first abdominal segment) ligations were carried out using waxed dental floss. Head ligated animals had the anterior portion of the head capsule and brain-retrocerebral complex removed; thoracic ligated animals had the head and thoracic regions removed (i.e., these animals were essentially isolated abdomens). All injections into head or thoracic ligated animals were given via the abdominal prolegs using 10 ul Hamilton syringes and the injection sites ligated with waxed dental floss; untreated and sham injected animals served as controls. RH 5849 was administered into the animals as single or double injections on day 3 (plus 4 hr) and day 4 (plus 0 hr) of the last larval stadium; injection volumes were all less than 10 ul per animal. Concentrations of RH 5849 injected ranged from a total of .02 to 3 nmol/g weight of animal.

Tissue Dissection and Homogenization. Midgut from gate II larvae was dissected in a lepidopteran Ringer's at 4°C (25). Homogenates of midgut were made at 10 to 100 mg/ml in sodium phosphate buffer (50 mM, pH 7.5, containing 250 mM sucrose) using a Potter-Elvehjem tissue grinder with a motor driven Teflon pestle (275 rpm, 20 strokes, 0-4°C).

Ecdysone 20-Monooxygenase Assay. Ecdysone 20-monooxygenase activity was detected and quantified in the midgut using a radioassay (17). For the assay, 0.05 ml aliquots of midgut homogenate (containing from 0.5 to 5 mg tissue equivalent)were added to 0.05 ml aliquots of 0.05 M sodium phosphate buffer, pH 7.5, containing [23,24-^{3}H] ecdysone (0.5 to 92.8 ng; 1.2 to 70 Ci/mmol; 0.011 to 2.0 x 10^{-6}M assay concentration) and NADPH (1.6 x 10^{-3}M assay concentration); some assays also contained RH 5849 (1.0 x 10^{-7} to 1.0 x 10^{-3}M assay concentration). Assay incubations were for 30 min at 30°C with constant agitation. All assays were run in duplicate with zero time controls and were terminated by the addition of 1.5 ml ethanol. Following termination, assay mixtures were centrifuged at 10,000g for 10 min and 0.15 ml aliquots of the assay supernatant plus 2 ug each of cold carrier ecdysone and 20-hydroxyecdysone were evaporated to dryness. The residues were redissolved in methanol and streaked on analytical thin layer chromatography (TLC) plates (0.25 mm silica gel 60, F-254; E. Merck, Darmstadt, Germany). The plates were developed in a solvent system of chloroform: 95% ethanol (4:1, v/v) and the ecdysone and 20-hydroxyecdysone bands visualized under short wavelength UV light. The ecdysteroid bands were scraped into scintillation vials, resuspended in scintillation fluid and counted using a Beckman Model 3801 scintillation counter (^{3}H counting efficiency, 65%). Control ecdysone 20-monooxygenase activity was expressed as pg 20-hydroxyecdysone (20-HE) formed/min/mg tissue (±SEM).

RESULTS AND DISCUSSION. The insecticide RH 5849 (1,2-dibenzoyl-1-*tert*-butylhydrazine) has been reported to mimic the actions of the naturally occurring insect molting

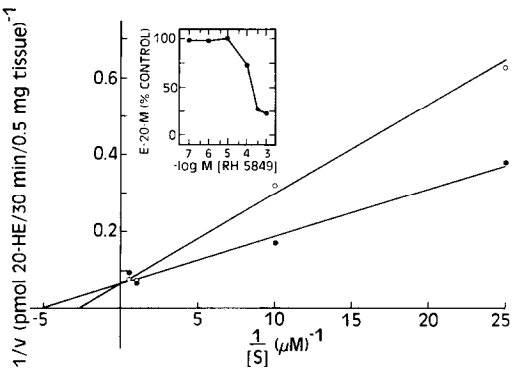

Fig. 1. Effects of RH 5849 on midgut ecdysone 20-monooxygenase (E-20-M) activity from day 5 gate II last instar larvae of *Manduca sexta*. Top inset depicts dose-dependent inhibition of midgut monooxygenase activity by RH 5849 (1 x 10^{-7}M to 1 x 10^{-3}M assay concentration); control monooxygenase activity, 430 (±07) pg 20-hydroxyecdysone (20-HE) formed/min/mg tissue (n = 6 for each data point). The kinetics of the RH 5849 inhibition of midgut ecdysone 20-monooxygenase activity is depicted in the Lineweaver-Burk plot. The RH 5849 concentration used in the kinetics experiments was 2.5 x 10^{-4}M; line fitting was by linear regression analysis (r = .992); n = 5 to 10 assays for each data point.

hormones under both *in vitro* and *in vivo* conditions (21, 22, 26, 27). Since the most dramatic ecdysonergic activities of RH 5849 appear to occur in lepidopterans, we decided to examine the effects of this compound on midgut ecdysone 20-monooxygenase activity in the tobacco hornworm, *Manduca sexta*.

Radioassay quantification of midgut ecdysone 20-monooxygenase activity following incubations of tissue homogenates with increasing concentrations of RH 5849 revealed that the ecdysone agonist inhibited the steroid hydroxylase activity in a dose-dependent fashion (Fig. 1, inset). The amount of RH 5849 required to elicit a 50% inhibition (I_{50}) of the midgut monooxygenase activity was graphically estimated to be about 2 x 10^{-4}M assay concentration. This finding is in essential accord with the pharmacological action of RH 5849 as an ecdysone agonist in that 20-hydroxyecdysone has been previously reported to inhibit under *in vitro* conditions and in a dose-dependent fashion the ecdysone 20-monooxygenase activity in abdomens of adult female *Aedes aegypti* (15), in *Drosophila melanogaster* third instar larvae (28), and in *Manduca sexta* fifth instar larval fat body (7). In these latter instances, however, the I_{50}'s for 20-hydroxyecdysone inhibition of enzyme activity were one to two orders of magnitude smaller (approximately, 1 x 10^{-5}M, *Aedes*; 1 x 10^{-6}M, *Drosophila*; and 2 x 10^{-5}M, *Manduca*) than that of RH 5849 in this study.

The kinetics of the RH 5849 inhibition of *M. sexta* ecdysone 20-monooxygenase activity was assessed by incubating midgut homogenates in the presence of increasing amounts of radiolabelled ecdysone (up to 2 x 10^{-6}M assay concentration) and a fixed concentration of RH 5849 (2.5 x 10^{-4}M assay concentration) near its solubility limit in the assay medium. Double reciprocal plots of the midgut monooxygenase activity in the presence and absence of RH 5849 revealed that this hydrazine derivative elicited an apparent competitive inhibition of the steroid hydroxylase activity (Fig. 1). The apparent inhibition constant (K_i) of RH 5849 was determined to be about 3.0 x 10^{-4}M. This finding provides an additional line of evidence in support and extension of the

ecdysonergic activities of RH 5849 inasmuch as earlier studies have demonstrated that 20-hydroxyecdysone is a competitive inhibitor of ecdysone 20-monooxygenase activity in *Manduca sexta* fat body, apparent K_i of 2.7 x 10^{-5}M (7); *Locusta migratoria* Malpighian tubules, apparent K_i of 7.5 x 10^{-7}M (6); and *Calliphora erythrocephala* whole body, apparent K_i of 2.1 x 10^{-7}M (29).

Under *in vitro* conditions RH 5849 seems to be a less potent inhibitor of ecdysone 20-monooxygenase activity on a per molar basis than 20-hydroxyecdysone, and earlier studies with RH 5849 have reported similar findings with other insect systems under *in vitro* conditions (21, 22, 26, 27, 30). Strikingly, just as 20-hydroxyecdysone appears to be more effective than RH 5849 at competitively inhibiting ecdysone 20-monooxygenase activity (presumably by competitively displacing radiolabelled ecdysone from its cytochrome P-450 binding site on the enzyme) so also has 20-hydroxyecdysone been reported to be more effective than RH 5849 at displacing the radiolabelled ecdysteroid ponasterone A from the cytosolic ecdysteroid receptor in Drosophila K_c cells (21, 27).

In contrast to the lower efficacy of RH 5849 under *in vitro* conditions, under *in vivo* conditions (particularly with lepidopterans) it has been demonstrated that this ecdysone agonist is more effective than the natural molting hormones at eliciting ecdysteroid mediated events (22, 27). This is presumably a reflection of a lowered metabolism or excretion of RH 5849 in comparison to 20-hydroxyecdysone.

Given the reported efficacy of RH 5849 under *in vivo* conditions in lepidopteran systems, it was of interest to examine the effects of this hydrazine compound on midgut ecdysone 20-monooxygenase activity in intact last instar larvae of *Manduca sexta*. Previous studies have demonstrated that ecdysone and 20-hydroxyecdysone under *in vivo* conditions elicit dramatic increases in the ecdysone 20-monooxygenase activity in *Manduca sexta* larval midgut (17), in *Locusta migratoria* larvae (18, 31) and in *Musca domestica* larvae (19, 20). In last instar larvae of *Manduca sexta*, the increases in midgut ecdysone 20-monooxygenase activity are quite dramatic (approximately a 50-fold increase from a basal level of 5-10 pg 20-hydroxyecdysone formed/min/mg tissue up to 350-450 pg 20-hydroxyecdysone formed/min/mg tissue) and temporally coincident with the onset of the wandering stage on day 5 of the last larval stadium of

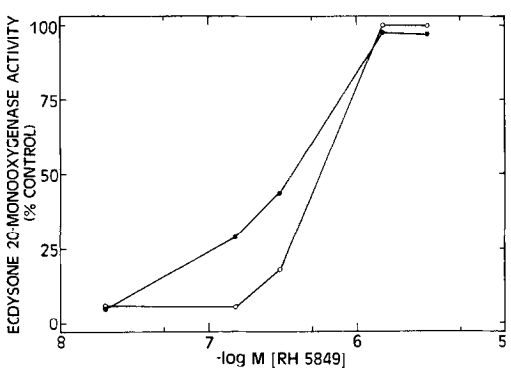

Fig. 2. Day 5 midgut ecdysone 20-monooxygenase activity in animals head ligated (closed circles) or thoracic ligated (open circles) on day 3 of the last larval stadium and injected with increasing amounts of RH 5849. Untreated day 5 control midgut monooxygenase activity was 412 (±19) pg 20-hydroxyecdysone (20-HE) formed/min/mg tissue (n = 2 to 4 assays for each data point).

gate II animals (12, 17). Although the exact mechanisms of how ecdysone and 20-hydroxyecdysone elicit increases in ecdysone 20-monooxygenase activity remain to be determined, it appears that the increases in enzyme activity are elicited by these specific steroids (and not by inactive ecdysteroid analogs, cholesterol or the cytochrome P-450 inducer phenobarbital) and are predicated on events occurring at both the transcriptional and translational levels.

When RH 5849 was injected into competent head or thoracic ligated pre-wandering last instar larvae of *Manduca sexta*, it was found that this compound elicited the 50-fold increase in midgut ecdysone 20-monooxygenase activity which would normally occur with the onset of the wandering stage (Fig. 2). The concentration of RH 5849 required to elicit the 50-fold increase in midgut ecdysone 20-monooxygenase activity in all of the animals (E_{100}) was graphically estimated at 1.5 x 10^{-6}M, and the amount required to elicit the same increase in midgut enzyme activity in 50% of the animals (E_{50}) was graphically estimated at 3.7 x 10^{-7}M and 5.6 x 10^{-7}M for head and thoracic ligated animals, respectively. By contrast, it has been demonstrated previously in our laboratory that injections of ecdysone or 20-hydroxyecdysone up to 1.8 x 10^{-5}M and 3.5 x 10^{-5}M, respectively, could elicit the 50-fold increase in midgut ecdysone 20-monooxygenase activity in only about 80% of the competent head or thoracic ligated pre-wandering stage larvae (17). Similarly, the $E_{50's}$ for ecdysone and 20-hydroxyecdysone were previously determined to be about 8 x 10^{-6}M and 1.8 x 10^{-5}M, respectively, for both head and thoracic ligated animals (17). It would appear, therefore, that RH 5849 can substitute for ecdysone or 20-hydroxyecdysone as the signal responsible for eliciting the 50-fold increase in midgut ecdysone 20-monooxygenase activity, and on a per molar basis RH 5849 is about 20 times more effective than ecdysone and about 50 times more effective than 20-hydroxyecdysone.

In conclusion, the data from this study demonstrate that RH 5849 behaves as an ecdysone agonist with respect to its pharmacological actions on ecdysone 20-monooxygenase activity both *in vitro* and *in vivo*. Our data support and extend, accordingly, the potential usefulness of RH 5849 as a pharmacological probe for further investigating the actions of ecdysteroids and their role(s) in the regulation of cytochrome P-450 dependent ecdysteroid monooxygenases.

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