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Effects of the adenylate cyclase activator forskolin and its inactive derivative 1,9-dideoxyforskolin on insect cytochrome P-450 dependent steroid hydroxylase activity

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Abstract. The adenylate cyclase activator forskolin and its pharmacologically inactive derivative 1,9-dideoxyforskolin were found to inhibit in a dose-dependent fashion the ecdysone 20-monooxygenase activity associated with wandering stage larvae of *Drosophila melanogaster* and fat body and midgut from last instar larvae of the tobacco hornworm, *Manduca sexta*. The concentrations of these labdane diterpenes required to elicit a 50% inhibition of the cytochrome P-450 dependent steroid hydroxylase activity in the insect tissues ranged from approximately 5×10^{-6} to 5×10^{-4} M.
Key words. Adenylate cyclase; cytochrome P-450; ecdysone 20-monooxygenase; forskolin; steroid hydroxylase.

Ecdysone (2β, 3β, 14α, 22R, 25-pentahydroxy-5β-cholest-7-en-6-one) is the secretory product of the insect prothoracic glands (the glandular source of molting hormone) and 20(R)-hydroxyecdysone is the more active metabolite of ecdysone and the predominant hemolymph ecdysteroid during most critical phases of postembryonic development[1]. Not surprisingly, the steroid hydroxylase enzyme system responsible for the conversion of ecdysone to 20-hydroxyecdysone, ecdysone 20-monooxygenase (EC 1.14.99.22), has been studied extensively both for the intrinsic importance of the reaction it catalyzes and as a model for understanding other insect steroid hydroxylases such as those in the prothoracic glands responsible in large part for the biosynthesis of ecdysone from cholesterol or plant sterols[1-3].

Several studies have revealed that ecdysone 20-monooxygenase is an NADPH requiring cytochrome P-450 dependent steroid hydroxylase system similar to the vertebrate cholesterol side chain cleavage system[4-9]. Additional studies have demonstrated that ecdysone 20-monooxygenase activity fluctuates dramatically and in a tissue specific fashion during the insect life cycle[10-17]. Like the vertebrate P-450 dependent steroid hydroxylases[18], the regulation of this insect enzyme system is probably complex and tissue specific. Ecdysone, 20-hydroxyecdysone and the ecdysone agonist RH 5849 have all been shown to enhance ecdysone 20-monooxygenase activity[17,19-21]. Other studies have reported that cyclic adenosine monophosphate (cyclic AMP) and factors which affect its metabolism may contribute to the regulation of ecdysone biosynthesis[26-29]. Accordingly, in the present study we examined the effects of the labdane diterpenes[30] forskolin (7β-acetoxy-8, 13-epoxy-1α, 6β, 9α-trihydroxylabd-14-en-11-one), an adenylate cyclase activator, and its inactive derivative 1,9-dideoxyforskolin on insect ecdysone 20-monooxygenase activity.

Materials and methods

Animals. The animals used in these studies were wandering stage third instar larvae of the Canton S strain of *Drosophila melanogaster*; and day 4 and day 5 (wandering stage) gate II fifth instar larvae of the tobacco hornworm, *Manduca sexta*. Animals were reared and staged as previously described[31,32].

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 (Scinti Verse E) were purchased from Fisher Scientific Co., Cleveland, OH; forskolin, its biologically active analog 7-O-hemisuc-

cinyl-7-deacetyl-forskolin, and its inactive analog 1,9-dideoxyforskolin were purchased from Calbiochem. Corp., San Diego, CA.

Tissue preparation and homogenization. Fat body from day 4 and midgut from day 5 gate II fifth instar larvae of *Manduca sexta* were dissected in a lepidopteran Ringer's[33] at 4 °C. Homogenates of the above tissues were made at 20 mg/ml for midgut and 100 mg/ml for fat body 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). Homogenates of wandering stage third instar larvae of *Drosophila melanogaster* were made at a concentration of 33 mg/ml in 100 mM sodium phosphate buffer (pH 7.5, 250 mM sucrose).

Ecdysone 20-monooxygenase assay. Ecdysone 20-monooxygenase activity was detected and quantified using a radioassay. For the assay, 0.05-ml aliquots of insect or tissue homogenate (containing 1.65 mg tissue equiv. of *Drosophila*, 5 mg tissue equiv. of *Manduca* fat body, or 1 mg tissue equiv. of *Manduca* midgut) were added to 0.05-ml aliquots of respective homogenization buffer (minus sucrose) containing [23,24-^{3}H]-ecdysone (from 3.3×10^{-8} to 3.3×10^{-7} M assay concentration; 1.4 to 40 Ci/mmol), NADPH (1.6×10^{-3} M assay concentration) and varying concentrations of forskolin, 7-O-hemisuccinyl-7-deacetyl-forskolin, or 1,9-dideoxyforskolin (1×10^{-7} to 1×10^{-3} M assay concentration). Incubation was 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, the assay mixtures were centrifuged at 10,000 g for 10 min and 0.15-ml aliquots of the assay supernatant plus 2 µg each of ecdysone and 20-hydroxyecdysone standards were evaporated to dryness. The residues were redissolved in methanol, and streaked on analytical thin layer chromatography 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 were 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%). Based on the total recovery of radiolabel (95–100%) in the ecdysone and 20-hydroxyecdysone bands compared to zero time controls, there was no evidence to suggest the formation of any metabolites of ecdysone other than 20-hydroxyecdysone under assay conditions with and without forskolin or its derivatives. Control ecdysone 20-monooxygenase activity was expressed as pg 20-hydroxyecdysone formed/min/mg tissue equiv.; forskolin and 1,9-dideoxyforskolin effects on ecdysone 20-monooxygenase activity were expressed as percent of control (±SEM).

Results and discussion

The adenylate cyclase activator forskolin[30] was found to inhibit in a dose dependent fashion the ecdysone 20-monooxygenase activity associated with wandering stage larvae of *Drosophila melanogaster*, and the fat body and midgut of *Manduca sexta* (fig. 1). A similar dose dependent profile of enzyme inhibition was obtained in all the insects with 1,9-dideoxyforskolin, a pharmacologically inactive derivative of forskolin (fig. 2). To the best of our knowledge, this is the first demonstration that forskolin and 1,9-dideoxyforskolin, or labdane diterpenes in general, can directly affect cytochrome P-450 dependent steroid hydroxylase activity.

At lower concentrations, 10^{-7} to 10^{-6} M, neither forskolin nor 1,9-dideoxyforskolin elicited any significant level of inhibition of the steroid hydroxylase activity in any of the insects (figs 1 and 2). As the concentration of forskolin was raised to 10^{-5} M the ecdysone 20-monooxygenase activity in *D. melanogaster* was significantly lowered to about 36% of control activity, whereas the activities of *M. sexta* fat body and midgut were lowered somewhat less to 60% and 58% of control activity, respectively (fig. 1). In contrast to forskolin, raising the concentration of 1,9-dideoxyforskolin in the assays to 10^{-5} M resulted in a less significant lowering of the ecdysone 20-monooxygenase activity to 79%, 93%, and 72% of the controls in *D. melanogaster*, and *M. sexta* fat body and midgut, respectively (fig. 2).

Increasing the concentration of forskolin to 10^{-4} to 10^{-3} M in the assays resulted in a significant lowering of

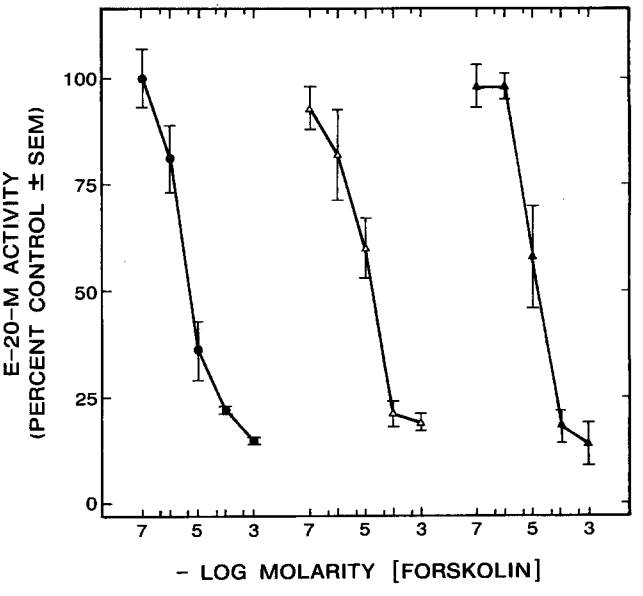

Figure 1. Effects of increasing concentrations of forskolin on the ecdysone 20-monooxygenase (E-20-M) activity in homogenates of wandering stage third instar larvae of *Drosophila melanogaster* (closed circles); day 4 fat body (open triangles) and day 5 midgut (closed triangles) from gate II fifth instar larvae of *Manduca sexta*. E-20-M activities are expressed as percent of control and each value is the mean (±SEM) of 5 to 12 determinations in duplicate. Control E-20-M activities were: *D. melanogaster*, 9 pg 20-hydroxyecdysone formed/min/mg tissue: *M. sexta* fat body, 41 pg 20-hydroxyecdysone formed/min/mg tissue; *M. sexta* midgut, 358 pg 20-hydroxyecdysone formed/min/mg tissue.

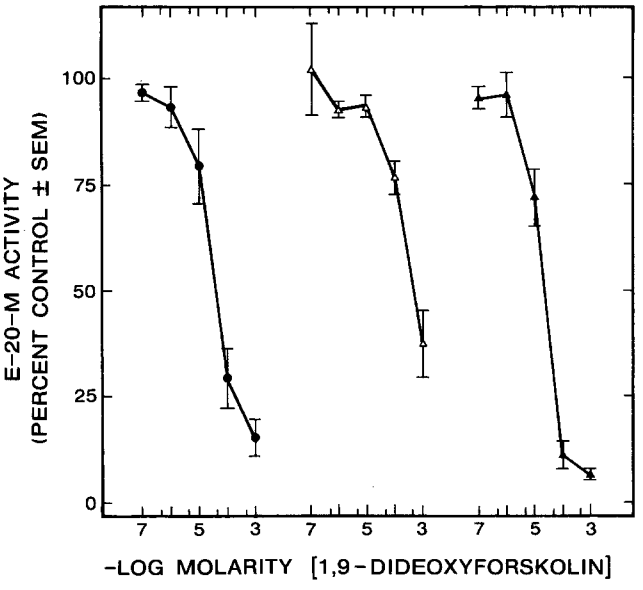

Figure 2. Effects of increasing concentrations of 1,9-dideoxyforskolin on the ecdysone 20-monooxygenase (E-20-M) activity in homogenates of wandering stage third instar larvae of *Drosophila melanogaster* (closed circles); day 4 fat body (open triangles) and day 5 midgut (closed triangles) from gate II fifth instar larvae of *Manduca sexta*. E-20-M activities are expressed as percent of control and each value is the mean (±SEM) of 5 to 12 determinations in duplicate. Control E-20-M activities were: *D. melanogaster*, 8 pg 20-hydroxyecdysone formed/min/mg tissue; *M. sexta* fat body, 48 pg, 20-hydroxyecdysone formed/min/mg tissue; *M. sexta* midgut, 340 pg 20-hydroxyecdysone formed/min/mg tissue.

the ecdysone 20-monooxygenase activity in all the insects to approximately 15–20 % of control activity; similar levels of inhibition were also noted (data not shown) with the biologically active analog 7-O-hemisuccinyl-7-deacetyl-forskolin. The concentration of forskolin required to elicit a 50% (I_{50}) inhibition of steroid hydroxylase activity was determined to be about 5×10^{-6} M for *D. melanogaster* and 2×10^{-5} M for *M. sexta* fat body and midgut.

Although 1,9-dideoxyforskolin was a less effective inhibitor than forskolin on a per molar basis, 1,9-dideoxyforskolin at 10^{-3} M was found to elicit significant levels of steroid hydroxylase inhibition in all the insects; 15%, 37%, and 7% of control for *Drosophila* and *Manduca* fat body and midgut enzyme activities, respectively (fig. 2). The I_{50}'s for 1,9-dideoxyforskolin were determined to be approximately 5×10^{-5} M, 5×10^{-4} M and 3×10^{-5} M, for *Drosophila* and *Manduca* fat body and midgut, respectively.

The findings of this study that both forskolin and 1,9-dideoxyforskolin can inhibit insect ecdysone 20-monooxygenase activity suggest that such inhibition is a direct function of a shared chemistry of these labdane compounds and independent of any adenylate cyclase involvement. Earlier studies have also demonstrated that several effects of forskolin are independent of adenylate cyclase activation, including its actions on voltage gated K^+ channels in pheochromocytoma cells[34] and acetylcholine receptor desensitization in skeletal muscle[35]. Accordingly, the use of forskolin as a pharmacological probe for determining the involvement of cyclic AMP in the regulation of ecdysone 20-monooxygenase activity[29] or ecdysteroidogenesis in general[22–25] must be called into question, unless other criteria are incorporated into the experimental design to circumvent possible misinterpretation of the data[22–28].

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