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Ecdysteroid Binding Activity in Embryos of Drosophila melanogaster

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Ecdysteroid binding proteins have been found in nuclei of Drosophila melanogaster embryos. Comparison of results derived from Scatchard analysis, analogue binding competition, and sucrose gradient centrifugation has revealed no significant differences between the properties of the putative embryonic receptor and those of the receptor found in imaginal disks or K_c cells.

Key words: Drosophila embryos, imaginal discs, ecdysteroid receptor, 20-hydroxyecdysone, ponasterone A

The steroid hormone 20-hydroxyecdysone acts throughout the life cycle of Drosophila to mediate morphogenesis, reproduction, and cuticle production. At each life stage—embryo, three larval instars, pupa, and adult—different tissues respond to this hormone in the manner characteristic for that tissue at that particular developmental stage. For example, 20-hydroxyecdysone mediates the synthesis of five cuticles in the life cycle. First, the larval epidermal cells produce three successive cuticles; the first and second larval cuticles have a very similar protein composition, but the third larval cuticle contains quite different proteins. Next, adult epidermal tissue set aside early in embryogenesis as imaginal disks differentiates and produces first the pupal and then the adult cuticle protein sets. The syntheses of the last two larval, the pupal, and adult cuticles occur during periods of high ecdysteroid titer [1]. Although embryonic ecdysteroid titer data are far less complete, it is presumed that synthesis of the first larval cuticle in the embryo is ecdysteroid-mediated as well.

The hormone 20-hydroxyecdysone evidently acts through a protein receptor molecule which mediates the hormone's interaction with chromatin [2,3]. Since the same hormone is used throughout the life cycle to control many different events, clearly the competence of the target tissues to respond to that hormone varies during development. This variation in competence could result from the absence of receptors in particular tissues at particular times, from different ecdysteroid receptors at each life stage or in each tissue, or from other factors. Through careful correlation of ecdysteroid binding parameters with the biological properties of hormone response both in vivo and in vitro the ecdysteroid receptors that mediate the initiation of morphogenesis and the synthesis of the pupal cuticle in Drosophila imaginal disks

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have been identified [2]. Receptors described in a Drosophila K_c cell line have very similar properties to those of imaginal disks [3].

To increase our understanding of ecdysteroid action, we have begun a comparative study of ecdysteroid receptors in other target tissues. For technical reasons it is much easier to work with whole embryos containing embryonic larval epidermis, as well as other embryonic tissues, than larval or adult epidermis from other stages. A survey of ecdysteroid binding activity in a high-speed cytosol fraction and a nuclear extract (to be reported in detail elsewhere) found that the total detectable ecdysteroid binding is roughly constant during embryogenesis, although the distribution of competible binding between the nuclear and cytoplasmic fractions does vary over developmental time. We chose to work with 6-hr embryos because published titer data suggest that endogenous free hormone is relatively low at this time [4]. In these experiments we have analyzed the nuclear extract which contains greater than 70% of the binding activity at this developmental stage. We report here partial characterization of an ecdysteroid binder with properties similar to those of the imaginal disk receptor. This molecule may be an embryonic ecdysteroid receptor.

MATERIALS AND METHODS

Embryo and Disk Collection

An Oregon R strain of D melanogaster maintained in mass culture for 15 years was used for all experiments. Embryos were collected over a 3-hr period, held for 3 hr at 25°C, collected on wire mesh filters, and dechorionated in a 50% solution of Clorox in distilled water. The dechorionated embryos were rinsed thoroughly with deionized water, checked under a dissecting microscope for proper staging, and then either frozen at −70°C or used immediately. No difference in recoverable ecdysteroid binding activity was detectable in embryos frozen for up to 1 month. Imaginal disks were mass-isolated according to published procedures [5].

Tissue Fractionation

All procedures were performed at 0–4°C. Aliquots of 0.5–1.0 g of embryos were homogenized in a Dounce homogenizer in 1.0–3.0 ml of 0.01 M Tris, 1.5 mM EDTA, and 7 mM dithiothreitol (DTT), pH 7.4 (TES), until no unbroken cells were found under phase-contract microscopy. The homogenate was spun at 1,000g for 10 min. The final pellet was extracted 3–4 times for 7–10 min in 0.2–0.8 ml of 0.3 M KCl in TES with spinning at 3,000g for 10 min. The pooled KCl extracts were dialysed against 0.1 M TRIS, 1.5 mM EDTA, pH 7.4 (TE), for 2 hr. After dialysis DTT was added to a final concentration of 7 mM. Nuclear extracts of imaginal disks were prepared as previously described [2].

Hormone Binding Assays

Ponasterone A (PNA), an ecdysteroid analogue with high biological activity, was used for all assays. Aliquots of 90–200 μl nuclear extract were incubated with 3.7 nM (^{3}H)PNA (sp. act. 110 Ci/mmol) overnight at 0–4°C. Bound and free hormone were separated on BioGel P-10 columns, and radioactivity was determined as previously described [2]. Specific hormone binding was determined as the average of duplicate assays of radioactivity bound with (^{3}H)PNA alone less radioactivity bound in the presence of 100-fold excess unlabeled hormone.

Sucrose Gradients

Linear 5–20% sucrose gradients in 0.01 M Tris, 1.5 mM EDTA, 7 mM monothioglycerol, pH 7.4, with 0.1 or 0.3 M KCl were equilibrated overnight. Aliquots of 200 μl of a prebound, dialyzed nuclear extract were layered on the gradients and centrifuged in a Sorvall TV865 vertical rotor at 350,000g (62 K) for 3 hr at 0–4°C. The gradients were fractionated manually from the bottom of the tube. Fractions were counted in a xylene based scintillation fluid in a Searles counter. Protein standards were loaded as 1 mg in 200 μl. Monothioglycerol was omitted from protein standard gradients because of interference with protein determination by the method of Lowry.

RESULTS

Scatchard Analysis

Figure 1 shows a typical Scatchard [6] plot of specific binding of increasing concentrations of (^{3}H)PNA to aliquots of an 0.3 M KCl extract of embryonic nuclei. The equilibrium dissociation rate constant (K_D) determined by Scatchard analysis of four experiments is $6 \pm 2.9 \times 10^{-9}$ M. This value is in good agreement with the K_D estimates from both imaginal disks and K_c cell line [2,3].

Analogue Specificity of (^{3}H)PNA Binding

Holding the amount of (^{3}H)PNA constant and varying the concentration of unlabeled analogue included in the incubation mixture provides a means of measuring the effectiveness of an analogue in preventing or displacing the binding of (^{3}H)PNA. Molecules should compete with (^{3}H)PNA binding according to their relative affinity for the hormone binder. Such competition experiments utilizing ecdysone and 20-hydroxyecdysone have demonstrated that 20-hydroxyecdysone is a more effective competitor than ecdysone. Since the concentration of (^{3}H)PNA used was equivalent

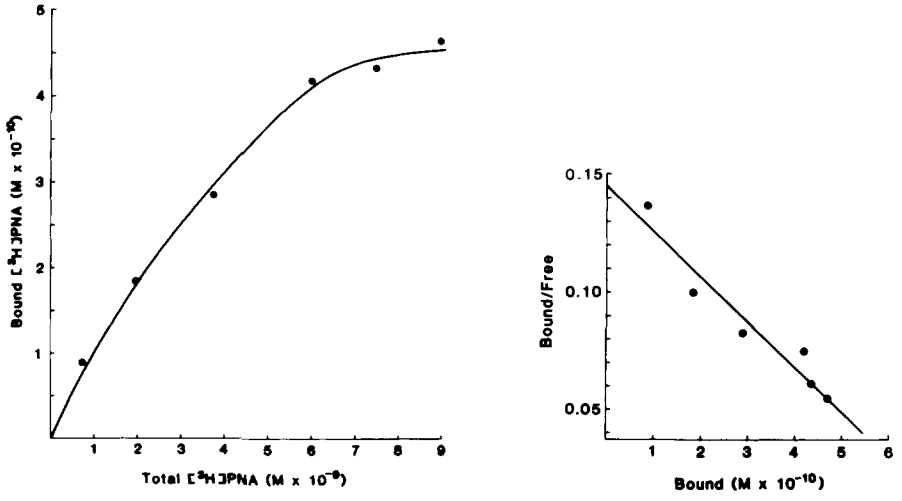

Fig. 1. a) Specific binding of (^{3}H)PNA to aliquots of an 0.3 M KCl TES extract of embryo nuclei in the presence of increasing concentrations of (^{3}H)PNA. b) Scatchard analysis of binding data from (a).

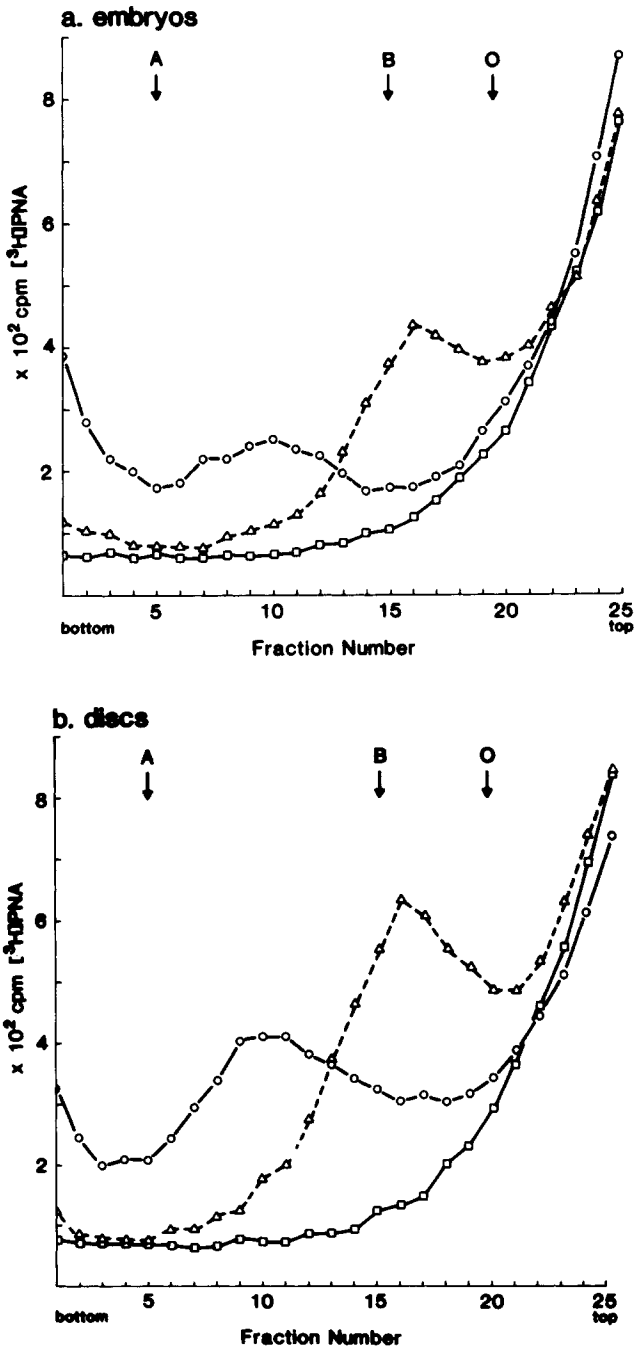

Fig. 2. Sucrose density gradient centrifugation of ecdysteroid bound to nuclear extract. a) Embryonic nuclei; b) imaginal disk nuclei. Nuclei were extracted in 0.3 M KCl in TES. The extract was dialyzed aginst TE, incubated with (^{3}H)PNA, dialyzed, and run on gradients containing 0.1 M KCl (○) or 0.3 M KCl (△). Equivalent specifically bound cpm were added and recovered from each gradient. A control sample incubated with (^{3}H)PNA + 100-fold excess unlabeled hormone was run on an 0.3 M KCl gradient (□). Sedimentation standards were aldolase (A, 7.35 S), bovine serum albumin (B, 4.35 S) and ovalbumin (O, 3.6 S).

TABLE I. Comparative Analogue Specificity

Tissue	20-Hydroxyecdysone K_D (M)	Ecdysone K_D (M)
Embryos	2×10^{-7}	5×10^{-5}
Disks [2]	5.2×10^{-8}	7.2×10^{-5}
K_c cells [3]	3.3×10^{-8}	5.5×10^{-6}

TABLE II. Sedimentation Values of the Hormone-Receptor Complex

Tissue	0.1 M KCl gradient	0.3 M KCl gradient
Embryo (nuclear)	6.0	4.3
Disk (nuclear)	5.95	4.3

to the K_D of PNA binding, the concentration of analogue required to displace 50% of the bound (^{3}H)PNA should be equivalent to the K_D for binding of that analogue to the receptor. The estimated K_D values for binding of ecdysone and 20-hydroxyecdysone to the embryonic binder are 5×10^{-5} M and 2×10^{-7} M, respectively. These results are comparable to those obtained for these analogues in imaginal disks and K_c cells (Table I).

Sucrose Density Gradients

The embryo-derived nuclear binding complex sedimented as a fairly broad band in an 0.1 M KCl gradient with a peak approximately 6 S, and as a somewhat narrower band in an 0.3 M KCl gradient with a peak at 4.3 S (Fig. 2a, Table II). The imaginal disk nuclear receptor sediments at 6 S in an 0.1 M KCl gradient and at 4.3 in an 0.3 M KCl gradient (Fig. 2b, Table II). Cytoplasmic fractions were not analyzed because of low levels of bound radioactivity.

DISCUSSION

Comparison of the data from experiments on the physical and kinetic properties of ecdysteroid binders from embryos, imaginal disks, and cell lines reveals many similarities and some minor differences. The value of the K_D of the embryonic binder ($6 \pm 2.9 \times 10^{-9}$ M) overlaps those for the imaginal disk receptor (3.3×10^{-9}) [2] and for the K_c cell receptor (3×10^{-9}) [3]. In all three systems, 20-hydroxyecdysone is a more effective competitor for (^{3}H)PNA than ecdysone. Because the binding was measured in crude extracts obtained from different tissues using comparable but not identical procedures, it is not possible at this point to critically evaluate differences in the apparent K_D values. We did note that the competition binding data for PNA and ecdysone in embryo extracts consistently gave curves with little scatter, whereas that for 20-hydroxyecdysone was consistently irregular. Although there was no detectable metabolism of labeled PNA (data not shown), it is possible that enzymes regulating free ecdysteroid concentration, known to exist in some insect embryos [7], contaminate our preparation. This could result in effective ecdysteroid concentrations different from the nominal amount added.

The S values of both the embryonic binder and imaginal disk receptor vary with the ionic strength of the gradient. The variance may be due to weak electrostatic interactions causing aggregation of the receptor with itself or other proteins, or to a conformational change in the ecdysteroid binder. The values of 4.3 S in an 0.3 M KCl gradient and 6.0 S in an 0.1 M KCl gradient are identical in disks and embryos, and virtually the same as those obtained for K_c cells [8]. However, the values of 4.2 S and 6 S obtained for K_c cells correspond to a cytoplasmic and nuclear receptor, respectively, rather than a sensitivity to ionic strength during centrifugation. The difference in these two values is again probably due to weak ionic interaction because freezing and thawing the nuclear fraction before running the gradient reduces the 6 S value to 4.2 S.

When one considers that the imaginal disk and K_c cell receptors and the embryonic ecdysteroid binders have been studied only in crude extracts, the similarities in ecdysteroid binding properties are quite striking. The embryonic ecdysteroid binder reported here may well function as a hormone receptor during embryogenesis. It would appear that a common receptor, or different gene products with similar hormone-binding domains, may be acting throughout the Drosophila life cycle. Detailed comparison of purified receptor from several sources, or identification and sequencing of receptor gene(s), will be necessary to determine the precise number and nature of the ecdysteroid receptor(s) acting during Drosophila development.

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