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Ecdysteroid receptor in *Chironomus thummi* (Diptera: Chironomidae)

PETER DEAK[1] and HANS LAUFER[2]

[1]Department of Genetics, Attila Jozsef University, P.O.Box 533, H-6726 Szeged, Hungary

[2]Department of Molecular and Cell Biology, U-125, The University of Connecticut, Storrs, CT 06268, USA

***Chironomus thummi*, ecdysteroid receptor, receptor concentration**

Abstract. An ecdysteroid receptor protein has been identified and characterized from whole body extracts of *Chironomus thummi* larvae. The kinetic and physical characteristics of this binding are consistent with the criteria established for steroid hormone receptors and are similar to those exhibited by the *Drosophila* ecdysteroid receptor. The binding data of cytosols obtained from different developmental stages (third and fourth stage larvae, pupae and adults) indicate only a single type of binding site which exists throughout the life cycle of the insect with an average K_d value of 5×10^{-9} mol/g of protein. Furthermore, single point determinations of the receptor concentration at these stages revealed that the receptor level fluctuates. This fluctuation in the receptor level corresponds well to the fluctuation in the ecdysteroid titer indicating that the number of receptors at any particular stage of development may be regulated by the presence of the moulting hormone.

INTRODUCTION

Ecdysteroids or moulting hormones (MH) are responsible in insects for regulating growth and development. It is believed, that similarly to vertebrate steroid hormones, ecdysteroids exert their effects by binding to an intracellular receptor protein (Bonner, 1982). However, the investigations of insect ecdysteroid receptors (EcR) have been limited mostly to *Drosophila melanogaster*. The existence and preliminary characterization of EcR have been shown in K_c cells (Maroy et al., 1978), in a tumorous cell line (Dinan, 1985), in imaginal discs (Yund et al., 1978) and in whole body extracts of animals with different developmental stages (Deak et al., 1988) of *Drosophila melanogaster*. The very similar physico-chemical characteristics of the EcR obtained from these studies indicate the existence of a single type of EcR with respect to hormone binding in this species. Our understanding of MH mode of action in *Drosophila* was improved with the cloning and molecular characterization of the gene encoding the EcR (Koelle et al., 1991). In an other set of experiments, a *Drosophila* ecdysteroid receptor was purified to apparent homogeneity and its DNA-binding properties were investigated (Luo et al., 1991). Subsequently it was demonstrated that the EcR gene encodes three receptor isoforms that have common hormone- and DNA-binding domains but they differ in their N-terminal regions (Talbot et al., 1993).

Because of the importance of EcR in hormone action, it is necessary to extend the investigations to other insect species. Chironomidae serve as a useful model system for studying processes involved in MH action during development, such as control of puff induction on salivary gland chromosomes (Clever & Karlson, 1960; Laufer & Calvet, 1972)

or the regulation of specific gene expression (Vafopoulou-Mandalos & Laufer, 1984a,b). Endogenous MH was bound and localized to ecdysone-sensitive loci of polytene chromosomes in *Chironomus tentans* (Gronemeyer et al., 1981). It was established that the chromosomal component to which the MH had been bound corresponds to the EcR (Gronemeyer et al., 1981) however, it was not investigated further. The presence of an ecdysteroid specific binding protein was demonstrated in epithelial tissue culture cells of *C. tentans* (Turberg et al., 1988), and it was shown that this protein also exhibits high affinity to DNA (Turberg et al., 1992). Recently, a cDNA clone encoding a protein homologous to the *Drosophila* EcR has been cloned, but the ligand specificity of this protein is yet unknown (Imhof et al., 1993).

In the present study we used ^{3}H-Ponasterone A (^{3}H-PNA) with high specific activity to demonstrate the existence of an ecdysteroid binding protein possessing steroid receptor characteristics in *Chironomus thummi* whole body extracts. Several physical characteristics of the receptor have been described and compared to those exhibited by the *Drosophila* EcR. Furthermore, the concentration of EcR was determined in different developmental stages by using saturating amount of ^{3}H-PNA. The fluctuating EcR levels obtained from these experiments indicate a developmental regulation of the receptor, similarly to fluctuations seen in MH concentrations.

MATERIALS AND METHODS

Animals

Laboratory stocks of *Chironomus thummi* were reared according to the procedures of Laufer & Wilson (1970) and the animals were staged as described by Vafopoulou-Mandalos & Laufer (1982).

Chemicals

Ecdysteroids (ecdysone, 20-hydroxyecdysone) were purchased from Simes (Milan, Italy). Ponasterone A was provided by D. Horn (Melbourne, Australia). ^{3}H-Ponasterone A was synthesized and purified as described by Sage et al. (1982) with a specific activity of 178 Ci/mmol. The purity of the hormones was verified by TLC and HPLC.

Cytosol preparation

Animals of different developmental stages were washed and weighted before homogenization. The samples were homogenized by a teflon/glass or a glass/glass homogenizer in a twofold volume of TE buffer (10 mM Tris, 1.5 mM EDTA, 7 mM 2-mercaptoethanol, pH 7.4). The homogenate was centrifuged (15,000 × g, 15 min, 4°C) and the supernatant was used as "S15 cytosol".

Receptor binding assay

100 µl aliquotes of cytosol were incubated with different amounts of ^{3}H-PNA for Scatchard analysis, and with 200 nM ^{3}H-PNA for determination of receptor titer. For determination of nonspecific association, parallel samples were incubated with ^{3}H-PNA and a 100-fold excess of unlabeled hormone. Bound ligand was separated from the free by a modification of the dextran-coated charcoal (DCC) method (McGuire, 1975). Each sample was incubated with 250 µl of 7% DCC (5 min, 4°C) then the suspension was centrifuged (10,000 × g, 5 min) and the radioactivity of 250 µl aliquotes of supernatants were measured by liquid scintillation spectrometry. Specific binding was calculated by subtracting counts of vials containing excess unlabelled hormone (nonspecific binding) from the counts of vials without excess (total binding). Data were normalized to the protein content of cytosols determined according to the colorimetric method of Bradford (1976).

Gel chromatography

Binding moieties of the cytosol were examined on a 0.6 × 25 cm Sephadex G-25 column (sample : volume ratio 1 : 50). 100–200 µl of samples preincubated with ^{3}H-PNA were run at 4°C in TE buffer without mercaptoethanol. Bound hormone was defined as hormone in the void volume (macromolecular fraction).

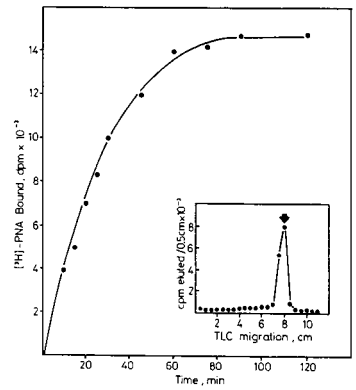

Fig. 1. Binding of ^{3}H-PNA (0.3 nM) by *C. thummi* larval extract at 22°C. The inset shows the radiochromatogram obtained after thin-layer chromatography (TLC) of a methanol extract of bound radioactivity. The radioactivity cochromatographed with an authentic ponasterone-A standard (arrow).

Free hormone was considered as that eluting coincidently with unreacted ^{3}H-PNA in the low molecular weight fraction.

RESULTS

Incubation of ^{3}H-PNA with 15,000 × g supernatant of *C. thummi* larval extracts resulted in binding of a certain amount of the label. The association reached equilibrium within 90 min at 23°C (Fig. 1). The identity of the bound radioligand was determined by methanol extraction and subsequent thin-layer chromatography. Figure 1 inset shows that 95–97% of radioactivity extracted ran coinsidently with an authentic PNA standard. This indicates that the cytosol does not metabolize PNA during the incubation time and the binding moiety associates with intact radioligand.

It was demonstrated previously that ecdysteroid receptors exist in both cytoplasmic and nuclear fractions of *Drosophila* imaginal discs (Yund et al., 1978) and in the K_c cell line (Maroy et al., 1978). In these studies, nuclear binding sites were solubilized by homogenization of discs and cells in TE buffer supplemented to 0.4 M KCl. Similar treatment of *Chironomus* samples with subsequent salt removal, failed to increase specific binding in the supernatant, and there was no detectable specific binding in the centrifugation pellet (containing the nuclear fraction) of the TE homogenate even after 0.4 M KCl extraction (data not shown). These results suggest the solubilization of total cellular binding sites with the homogenization and extraction procedure used here.

The macromolecular nature of the binding moiety was demonstrated by Sephadex G-25 chromatography of cytosol samples incubated with ^{3}H-PNA. Figure 2A shows that a

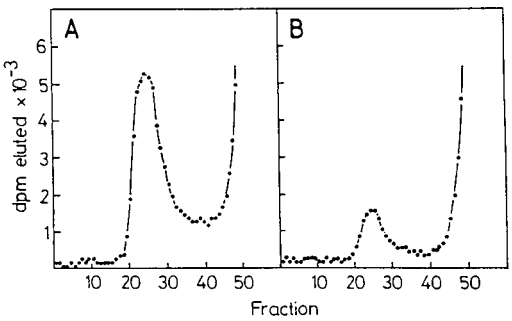

Fig. 2. Sephadex G-25 chromatography of cytosols from *C. thummi* larvae at 4°C. (A) Incubation of cytosol with ^{3}H-PNA (0.5 nM) for 90 min at 22°C. (B) Cytosol incubation as in A except that 1 ng of unlabeled PNA was added.

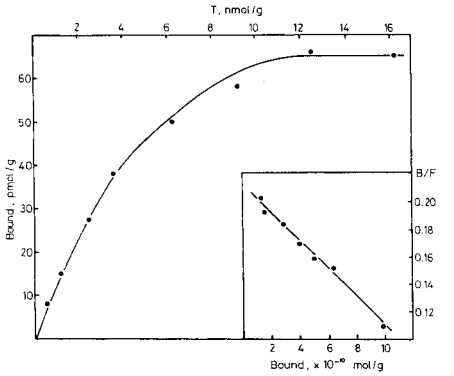

Fig. 3. Saturation analysis of ^{3}H-PNA binding in cytosol of *C. thummi* larvae. The cytosol was incubated with increasing amounts of ^{3}H-PNA for 90 min at 22°C. The specific binding (B), determined as described in Materials and methods, was plotted against the total (T) PNA concentration present in the assay. A Scatchard plot of the same data is shown in the inset. K_d = 5.0 nmol/g; B/F: bound to free ratio.

significant proportion of the radioactivity eluted in the void volume. This radioactivity was bound to macromolecules and could be decreased by excess unlabelled hormone (Fig. 2B) or by preincubation of the cytosol at 42°C for 30 min. (data not shown).

The saturability of the binding sites was titrated by incubation of cytosol samples with increasing amounts of ^{3}H-PNA. Figure 3 shows the saturation kinetics of cytosol isolated from fourth stage larvae. Scatchard analysis of the binding data (Fig. 3 inset) indicates only one class of binding sites present with a K_d value of 5×10^{-9} mol/g protein. Cytosols obtained from animals with different developmental stages possessed different binding capacities but similar dissociation constants with an average value of 5×10^{-9} mol/g protein (data not shown).

Specificity of the binding moiety for ligand was demonstrated by competition studies with unlabelled PNA, 20-hydroxyecdysone and ecdysone respectively. When aliquotes of cytosol were incubated with varying amounts of competitor ecdysteroids, effectiveness in replacement of ^{3}H-PNA was PNA > 20-hydroxyecdysone > ecdysone (Fig. 4.). At the 50% competition level, PNA was about 30-fold and 150-fold more efficient than 20-hydroxyecdysone and ecdysone, respectively.

The stability of the binding moiety was studied at 4°C and 23°C in the presence or absence of hormone (Fig. 5A). Incubating the cytosol with radioligand, the maximal binding was reached within 2 h at 23°C, after that the binding activity remained relatively constant up to 16–20 h, then it decreased with a half life of approximately 35 h. In contrast, if the cytosol was incubated with radioligand at 4°C, maximal binding was achieved within

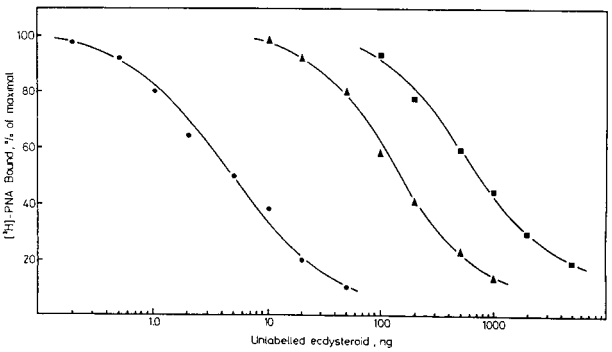

Fig. 4. Ecdysone (■), 20-hydroxyecdysone (▲) and PNA (●) inhibition of ^{3}H-PNA binding. Aliquots of *C. thummi* larval cytosol were incubated (90 min, 22°C) with increasing amounts of unlabeled ecdysteroids in the presence of ^{3}H-PNA (1.5 nM). The specific binding was determined as described in Materials and methods.

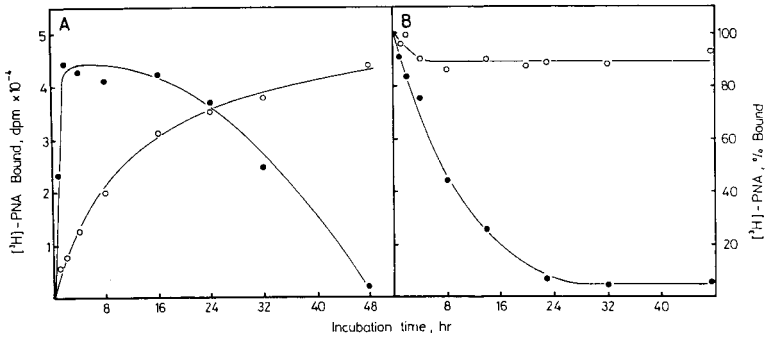

Fig. 5. Influence of storage on specific binding activity. Cytosols were incubated at 22°C (●) or at 4°C (○) either in the presence (A) or absence (B) of ^{3}H-PNA (1.5 nM). At times indicated, aliquotes were taken and specific binding was determined either immediately (A) or after incubation with ^{3}H-PNA for 90 min at 22°C (B).

40–48 h and it remained constant up to 70–76 h. These data indicate that the hormone binding activity of the cytosol is temperature dependent and it is very stable at 4°C. This was further supported by incubation of the cytosol without hormone at both temperatures (Fig. 5B). At 23°C, a 50% loss of binding activity occurred within 6–8 h and there was no detectable specific binding after 20–24 h. The binding moiety proved to be very stable at 4°C, even when maintained without hormone. Comparing the data presented in Figs 5A,B, it is obvious that the hormone binding has a stabilizing effect on the receptor.

For an estimate of receptor concentrations at different developmental stages, specific binding was measured in "duplicate tube" assays using saturating ^{3}H-PNA concentrations (see Materials and methods). The results are shown in Figure 6. Relatively low receptor levels were found in one day old third and fourth instar larvae and prepupae. Compared to these values, significantly higher receptor concentrations were found in three day old third instar larvae, 7–8 day old fourth instar larvae and two day old prepupae. Relatively high receptor levels were found in one and two day old pupae. The high receptor concentrations in these developmental stages coincide very well with the high ecdysone levels reported in these animals (Laufer et al., 1986).

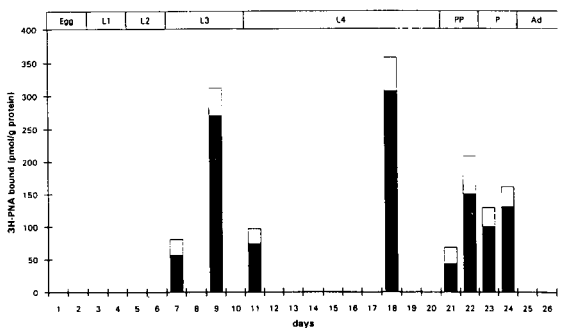

Fig. 6. Ecdysone receptor levels in different developmental stages of *C. thummi*. The receptor concentration was determined in „duplicate tube" assays as described in Materials and methods. Points represent the mean of 3 different experiments, open bars indicate SD.

DISCUSSION

The data available so far on EcR have been obtained mostly from different *Drosophila* sources: K_c cells, imaginal discs, embryos, larvae and adults. A better understanding of moulting hormone action is of great interest and here we extend studies to another species. In this paper, ^{3}H-PNA with high specific activity has been used to identify and characterize an ecdysteroid binding component from cell-free extracts of *Chironomus thummi*. The kinetic parameters – saturability, specificity and high affinity of binding reported here fulfill the criteria established for steroid hormone receptors, therefore, the hormone binder may function as EcR in the animals. The value obtained for the equilibrium binding constant (5.0 nmol/g protein) is in good agreement with the binding constant for the embryonic and larval receptors found in *Drosophila melanogaster* (Deak et al., 1988). The *Chironomus* receptor exhibits a ligand specificity similar to that from *Drosophila* (Maroy et al., 1978; Deak et al., 1988). The very consistent characteristics of the binding moiety derived from this study indicate the existence of only one class of hormone binding sites in this species. A similar situation was found in *Drosophila* (Deak et al., 1988) despite the presence of multiple receptor isoforms (Talbot et al., 1993). These results can be explained by assuming that the different receptor forms exhibit similar hormone binding activities. In fact, studies of EcR isoforms synthesized in yeast demonstrated that this is indeed the case (Talbot et al., 1993).

The level of the ecdysteroid binding receptor is developmentally regulated in all developmental stages investigated. Low receptor levels were found when the MH titers were low, and high receptor levels corresponded to stages with high MH levels. This consistent sequence of hormone and receptor level changes suggests the induction of the receptor by the hormone similarly to the process reported in *Drosophila* and in vertebrates (Deak et al., 1988; Syms et al., 1985). The change of receptor level can be an intermediate step between the hormone titer peak and the specific mRNA induction. Having shown the existence of an ecdysteroid receptor in *C. thummi* whole body extracts, its structure and function should be demonstrated. This can be achieved by cloning and expression of the gene encoding the receptor and/or by purification of the receptor.

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