Presence of membrane ecdysone receptor in the anterior silk gland of the silkworm, Bombyx mori : biochemical and molecular studies

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学位論文要旨

ABSTRACT

Nongenomic action of an insect steroid hormone, 20-hydroxyecdysone (20E), has been implicated in several 20E-dependent developmental events including the programmed cell death (PCD) of *Bombyx mori* anterior silk glands (ASGs), but no information is available for the mode of action. We provide several lines of evidence for a putative membrane receptor (mEcR) located in the plasma membrane of ASGs as an integral membrane protein. We showed that the putative mEcR could mediate the rapid increase in cAMP level in ASG cells, and might be involved in the activation of adenylyl cyclase through Gsα. The membrane fractions did not contain conventional EcR as revealed by western blot analysis using anti EcR-A antibody. The mEcR exhibited saturable binding for [^{3}H]ponasteroneA (K_d= 17.3 nM, B_{max}= 0.82 pmol/mg). Association and dissociation kinetics revealed that [^{3}H]ponA associated with and dissociated from mEcR within minutes.

We also defined the experimental conditions that allowed solubilization of mEcR from ASGs membranes with preserving 75.1% of the native binding activity. Dissociation of the protein/lipid/detergent mixed micelles, yielded protein/detergent

complexes with partial binding activity, which appeared enough to be adopted for protein purification. The present results clearly show that insect tissue membranes contain ecdysone receptors, and that the steroid membrane receptors are commonly present in insects and mammals.

Finally, we successfully raised a monoclonal antibody against the putative mEcR and obtained two GPCRs candidates from ASGs of *Bombyx mori*, which gave us the hope to step on the way for mEcR gene cloning.

INTRODUCTION

The holometabolous insect, *Bombyx mori,* belongs to order Lepidoptera, and its larvae undergo metamorphosis into pupae then into moths. At the pupal metamorphosis, most of larval specific tissues degenerate. The degeneration is known as programmed cell death (PCD). The silk gland is a larval-specific tissue that degenerates shortly after pupation. The anterior silk gland (ASG) is a mere duct composed of a single cell-layer consisting of about 250 cells. It forms silk filament out of the silk proteins. At the end of the larval stage, after the cocoon has been spun, the ASGs enter the process of PCD in response to the high hemolymph ecdysteroid concentration, which induces pupal metamorphosis. The PCD is induced by 20E in vitro and takes 120-144 h to be completed, at which time-point an oligonucleosomal ladder of DNA and apoptotic bodies appear. During the period with 20E, gene expression required for the completion of the PCD occurs during the first 8 h for transcription and in the first 18 h for translation. If the genomic theory of steroid action is applicable to the 20E-induced PCD, the 20E challenge for 18 h should be sufficient for full apoptosis. Nevertheless, withdrawal of 20E after 18 h and up to 42 h of culture interferes with the progression of apoptosis, and the extent of inhibition is reduced with delaying the time of 20E withdrawal. This suggests that effects of 20E during the period between 18 and 42 h are

not accompanied by gene expression but rather mediated by a nongenomic pathway, probably through a membrane receptor and second messenger. If this is the case, ASG plasma membranes should contain high-affinity binding sites for ecdysteroid. In the present study, we provide for the first time physiological and biochemical evidence for an ecdysone membrane receptor (mEcR) and its topological localaization. In addition we performed the full kinetic analysis required for the characterization of the newly identified ecdysteroid receptor in the membranes of insect cells (Chapter I).

We defined the experimental conditions that allowed solubilization of mEcR from ASGs membranes with preserving 75.1% of the native binding activity. Dissociation of the protein/lipid/detergent mixed micelles, yielded protein/detergent complexes with partial binding activity, which appeared enough to be adopted for protein purification (Chapter II).

RESULTS AND DISCUSSION

We provide several lines of evidence for a putative membrane receptor (mEcR) located in the plasma membrane of ASGs as an integral membrane protein. We showed that the putative mEcR could mediate the rapid increase in cAMP level in ASG cells, and might be involved in the activation of adenylyl cyclase through Gsα. The membrane fractions did not contain conventional EcR as revealed by western blot analysis using anti EcR-A antibody (Fig. 1). The membrane receptor exhibits a specific and saturable binding for [^{3}H]PonA with a K_d of 17.2 × 10^{-9} M (Fig. 2). This value is physiologically relevant to the prevailing hemolymph concentrations of 20E (ranging between 10^{-7} – 10^{-6} M) in the prepupal period when PCD is triggered *in vivo*. The association and dissociation kinetics indicate that PonA association with and dissociation from its binding sites are rapid, which is characteristic of the binding of several natural

compounds to their membrane receptors. The observed association constant (K_{obs}) was 0.9 ± 0.2 min^{-1}. The dissociation of PonA from the membranes occurs within 10 seconds with a dissociation constant (K_{off}) of 2.3 ± 0.5 min^{-1}. The calculated association rate constant (K_{on}) was 13.3×10^7 M^{-1} min^{-1}, and the estimated dissociation constant at equilibrium (K_d) was 17.5 nM. The saturation curve indicates the presence of a single high-affinity binding site and an apparent maximal number of binding sites of 0.82 pmol mg^{-1} protein. The obtained K_d value is supported by good accordance of the K_d with the estimated dissociation rate constant at equilibrium ($K_d = 17.5 \times 10^{-9}$ M). A second line of evidence to support the existence of a membrane receptor is that the binding affinity of PonA is less than that of 20E. The binding affinity of PonA to the nuclear receptor complex of EcR/USP is one to two orders of magnitude higher than that of 20E. The competition assay using the ASG membrane fractions shows that the binding affinity for PonA is one-fourth of that for 20E and that the values for non-steroidal ecdysone agonists are much lower than 20E, which totally differs from the binding characteristics of the conventional EcR. The competition analysis showed also that the IC_{50} values for three non-steroid agonists, RH-5849, tebufenozide (RH-5992) and methoxyfenozide (RH-2485) were much lower than those for PonA and 20E. The binding activity was in the order of 20E > PonA >> methoxyfenozide > tebufenozide > RH-5849.

For characterization of the novel mEcR, we tried the solubilization of functional mEcR from ASGs of *Bombyx mori* (Chapter II). However, there is no single detergent or solubilization scheme universally applicable to all membrane proteins, we defined the experimental conditions that allowed solubilization of mEcR from ASGs membranes with preserving 75.1% of the native binding activity. Dissociation of the protein/lipid/detergent mixed micelles, yielded protein/detergent complexes with partial binding activity, which appeared enough to be adopted for protein purification. Because of the extremely small amount of solubilized proteins obtained from the ASGs

membrane preparations and the inability to mass isolate the ASGs, we prepared membrane fractions from the fat body cells of the G0 larvae. The analysis for the fat body membrane fractions showed the presence of a single high-affinity binding site in each molecule, with an apparent K_d and B_{max} of 15.08 nM and 0.42 pmol mg^{-1} protein, respectively. Nevertheless the presence of mEcR in the fat bodies provide a possibility for purification and identification of mEcR from the mass-isolated fat bodies. Moreover, the presence of mEcR in the fat bodies indicates an equivocal presence in various tissues and its importance in understanding the effect of 20E at molecular levels.

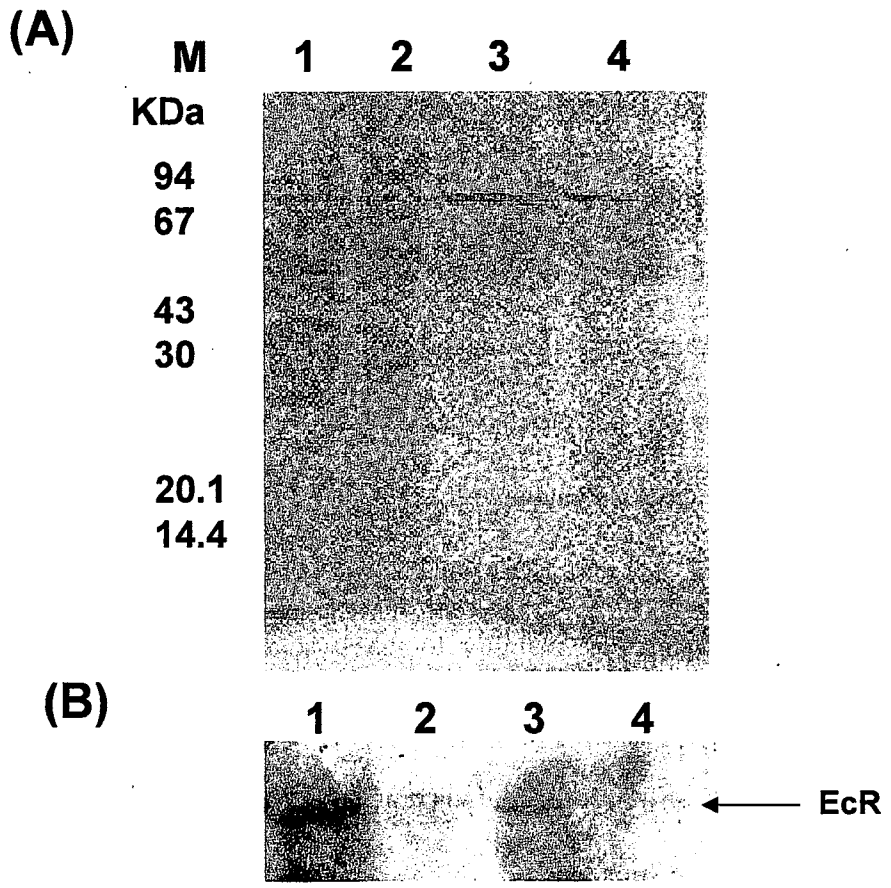

Fig. 1. Absence of conventional EcR in the membrane fractions. (A) Commassie brilliant Blue-stained 12 % SDS-PAGE gel. (B) Western blot for the identical gel using anti-EcR-A monoclonal antibody (1:100) as a first antibody and HRP-conjugated Protein A (1:1000) as a second antibody. Lane 1, total lysate; lane 2, membrane fraction; lane 3, nuclear extract. Samples for lane 1-3 were prepared from fresly dissected ASGs. Lane 4, membrane fraction prepared from the cultured ASGs used in the binding experiments. Twenty μg for each lane. M,protein molecular weight marker.

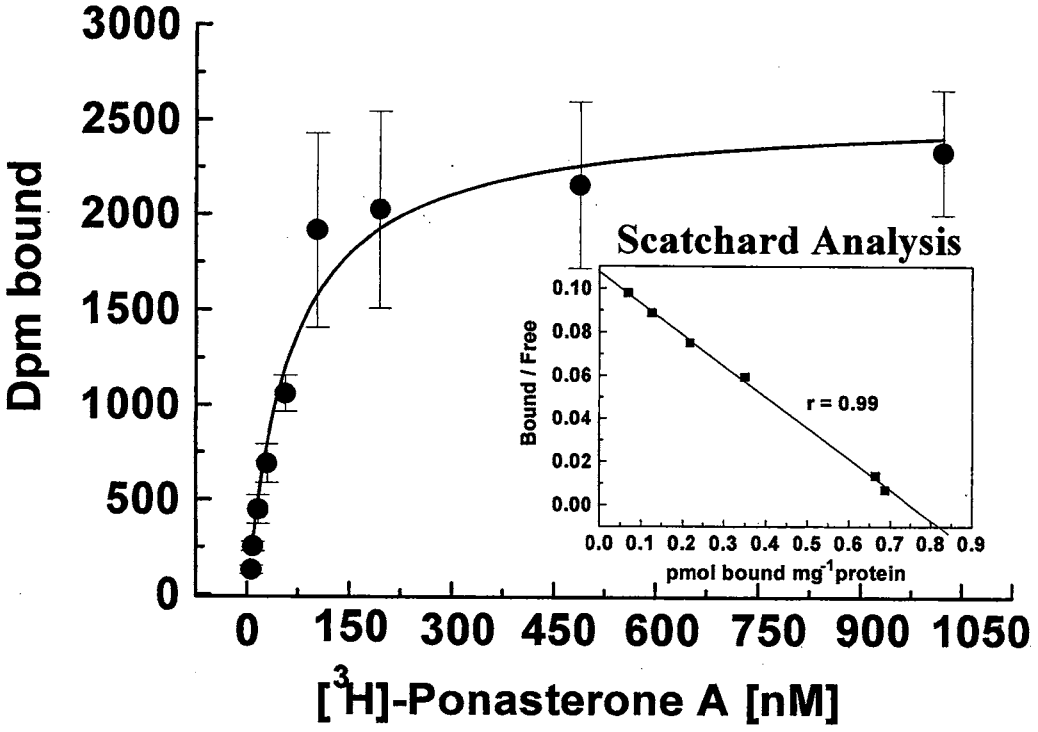

Fig. 2. PonA saturation analysis of ASG membranes. Membrane preparations (100 µg protein in 100 µl buffer) were incubated with increasing concentrations of [^{3}H]PonA at 25 °C for 10 min without or with a 1000-fold excess of unlabelled PonA. The data was fitted by nonlinear regression analysis. Inset is Scatchard analyses of the binding data. K_d = 17.3 nM; B_{max} = 0.82 pmol mg^{-1} protein. Each datum point is a mean ± S.D. (n = 3).

学位論文審査結果の要旨

昆虫の成長、脱皮、変態は一種類のステロイドホルモン、20-ヒドロキシエクジソン（20E）により調節されている。これまで20Eの初期作用はheterodimerの核受容体（EcR/USP）と結合し、遺伝子の転写調節にあるとされてきた。しかし、エルモギ君の所属する研究室ではこれまで20Eによる前部絹糸腺の予定細胞死において、核受容体を介した作用の他、恐らく膜受容体を介した作用経路もあることが示唆されてきた。エルモギ君は前部絹糸腺膜を材料として膜受容体の存在を証明することから研究を始めた。この分野は未知領域であり、まず膜受容体のホルモン結合活性を測定する系を確立し、それにより結合部位の生化学的特性を明らかにした。その結果、解離乗数は10nMオーダーでEcR/USPよりも優位に低いものの、体内20E濃度で十分活性化されうる解離常数を示した。たまエクジソンアゴニストとの結合はEcR/USPに対するよりも2桁弱かった事から、20Eの膜受容体の存在を補強するものであった。また、膜受容体のモノクローナル抗体の作成に成功し、これを用いて絹糸腺以外の組織で多量に入手できる脂肪体にも恐らく同一の膜受容体があることを示し、膜受容体の精製の道も開いた。本学位論文は、同一細胞が膜受容体と核受容体の双方を有し、これらを介して単一の細胞応答を調節するというステロイド作用機序の理解の基礎を与えるものであり、学位授与に十分な業績といえる。