A Novel Ecdysteroid Responsive Reporter Plasmid Regulated by the 5'-Upstream Region of the Drosophila melanogaster (Diptera: Drosophilidae) Acetylcholinesterase Gene

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Acetylcholinesterase activity induction by ecdysteroids is known in Drosophila melanogaster Kc cells, but its induction at the gene expression level has not been studied. A reporter plasmid was constructed by ligation of the D. melanogaster acetylcholinesterase gene (Ace gene) 5'-upstream 1.62 kb region to firefly luciferase cDNA. The plasmid was then introduced into Kc cells by the electroporation method for induction study. With the addition of 1.0 x 10^-8 M 20-hydroxyecdysone, a 4-fold induction of luciferase was observed 72 h after the hormone treatment, while 1.0 x 10^-6 M ecdysone was required to give a 5-fold induction. Dibenzoyl hydrazines (RH 5849 and RH 5992), which are known as non-steroidal ecdysteroid agonists, also induced a 3 to 4-fold induction, however, RH 5992 showed a higher induction potential than RH 5849 (as in other ecdysteroid assays). These results suggest that this novel ecdysteroid responsive reporter plasmid (system) might be useful for the evaluation of ecdysteroids and further analysis of the acetylcholinesterase induction mechanism by ecdysteroids.

Key words: ecdysteroid, acetylcholinesterase, Drosophila melanogaster, Kc cell, gene expression

INTRODUCTION

The Kc cell line originating from Drosophila melanogaster embryos has been extensively studied as a model system for the mode of action of ecdysteroids. Eserine sensitive acetylcholinesterase induction by 20-hydroxyecdysone was first shown in the Kc cell line (Kc-H subline) (CERHAS et al., 1977). Also, the French Kc cell line and Schneider line 3 showed both acetylcholinesterase induction and a morphological change of process protrusion (BERGER et al., 1978). The acetylcholinesterase of Kc-H cells was slowly induced by 20-hydroxyecdysone: a small induction was observed one day after the treatment, a more than 20-fold induction (compared with the hormone untreated cells) 2 days after treatment and a 50-fold induction was observed 3 days after treatment (CERHAS et al., 1977). However, original Kc cells showed rather moderate 4-fold induction after 3 days of culture with 1.0 x 10^-8 M of 20-hydroxyecdysone (WING, 1988).

The acetylcholinesterase gene of D. melanogaster was encoded by a single gene Ace and its DNA sequence has been elucidated (FOURNIER et al., 1989). No hitherto known ecdysteroid responsive elements have been found in the 5'-upstream of the Ace gene, and studies of the Ace gene at the level of gene expression have not been reported as yet.

In this paper, it is presented that the Kc cells, which were introduced with reporter plasmid constructed by ligation of the Ace gene upstream promoter region to the luciferase gene, show the induction of luciferase activity in response to ecdysteroids.
MATERIALS AND METHODS

Chemicals. 20-Hydroxyecdysone (98% purity) and ecdysone (95% purity) were purchased from Sigma Co. RH 5849 and RH 5992 were synthesized from N-benzoyl-N'-tert-butyl-hydrazine and N-4-ethyl-benzoyl-N'-tert-butyl-hydrazine, respectively, and 99% purity products were used (HSU, 1991; OIKAWA, 1994).

PCR amplification of the D. melanogaster 1.62 kb Ace gene upstream region. The Ace gene upstream region (−1,520 to +98) (FOURNIER et al., 1989) was amplified by the PCR method using Vent DNA polymerase. The PCR reaction included 1 μg of D. melanogaster Canton-S genomic DNA (Clontech Co.), 100 pmol of each primer, 20 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 100 μg/ml BSA, 400 μM deoxyribonucleotides and 5 units of Vent DNA polymerase (Biolabs Co.). The sequences of the primers were as follows: forward primer, 5'-GGGGGATTCGAGATGGAAT-3'; reverse primer, 5'-AACTGAAGCTTAAAAGGCATCGCAA-3'. The reaction mixture was overlaid with paraffin liquid, incubated at 94°C for 4 min, taken through 31 cycles of 1 min at 94°C, 1 min at 58°C, and 1 min 40 s at 74°C. After the reaction, the mixture was immediately chilled on ice and then treated with chloroform. PCR products were separated by electrophoresis through 1.0% agarose gel, and the 1.62 kb DNA fragments were purified from the gel using a GENECLEAN II kit (Bio 101 Co.).

Construction of pDACH-E-LUC plasmid. Pikkagene basic vector plasmid (pPGBV; Toyo Ink Co.) was digested with Bgl II and blunted with Klenow fragments. Gel-purified PCR products were ligated to the blunt-end plasmid by T4 DNA ligase at 15°C and transformed into Escherichia coli DH5 α competent cells. The right direction fragments containing clones were selected by sequencing with an Automated DNA Sequencer (Applied Biosystems Co.) using the Taq Dye Terminator method.

Transfection and induction of cultured cells by ecdysteroids. pDACH-E-LUC plasmids were prepared on a large scale by combination of the alkali lysis method and CsCl/ethidium bromide equilibrium centrifugation. Kc cells grown as late log phase were harvested by centrifugation for 5 min at 1,000 rpm (180 x g) at 4°C (RPR9-2 rotor; Hitachi Co.). Pellets were resuspended with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.3) at approximately 8.0 x 10⁶ cells/ml. The pDACH-E-LUC plasmid DNA was added (80 μg/ml) to the suspension which was gently mixed and kept on ice for 5 min. The DNA/cell mixture was then transferred to an ice cold electroporation cuvette (0.4 cm electrode distance) in 0.5 ml aliquots, and electroporation was carried out as a single shock at 0.5 kV with a 3 μF capacitance. The cells were diluted 20-fold in M3 (BF) medium supplemented with 2% fetal calf serum, and transferred to 6 cm diameter tissue culture plastic dishes in 2.5 ml aliquots and incubated with 2.5 μl of dimethyl sulfoxide containing various concentrations of ecdysteroids (dimethyl sulfoxide only for the control), at 25°C under 5% CO₂. To prepare cellular extracts, cells were washed once with PBS, and 250 μl of cell lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-N,N',N,N'-tetraacetic acid, 10% glycerol, 1% Triton X-100) was added to each dish. Aliquots of cellular extracts were used to measure luciferase activity and to determine protein concentration by the BCA protein assay (Pierce Co.) for normalization (MIKITANI, 1995 a). For each concentration, 2 to 4 dishes were used, and luciferase activity as well as protein concentration of the cellular extract were measured as a single determination. Each normalized luciferase activity was divided by the normalized luciferase activity of the control, and was defined as “Fold induction.”
Fig. 1. PCR amplified 1.62 kb 5′-upstream region of the *Drosophila melanogaster* acetylcholinesterase gene (A) and construction of the luciferase reporter plasmid pDACH-E-LUC (B). A, lane 1, DNA fragment standards; lane 2, PCR amplified products.

RESULTS AND DISCUSSION

The 5′-upstream 1.62 kb fragment of the *D. melanogaster* *Ace* gene, which contained a TATA box, was PCR amplified by Vent DNA polymerase (Fig. 1A). The luciferase reporter plasmid pDACH-E-LUC was constructed by insertion of this 1.62 kb fragment into pPGBV (Fig. 1B). The inserted 1.62 kb fragment was sequenced by the Taq Dye Terminator method with an Automated Sequencer (Applied Biosystems) and was identical to the reported sequence (FOURNIER et al., 1989).

The time dependence of luciferase induction in pDACH-E-LUC introduced Kc cells by 1.0 × 10⁻⁸ M of 20-hydroxyecdysone revealed a slow log phase-like increase and, 72 h after treatment, a 3.78 ± 0.01 (SD)-fold (*n* = 3) induction was achieved (Fig. 2). However, no induction of luciferase activity was observed in pPGBV introduced Kc cells with or without 20-hydroxyecdysone for 72 h.

The dose-response for luciferase induction in pDACH-E-LUC introduced Kc cells by 20-hydroxyecdysone 72 h after treatment was studied (Fig. 3A). No induction was observed at 1.0 × 10⁻⁹ M of 20-hydroxyecdysone, but at 3.0 × 10⁻⁸ M, an induction of greater than 3-fold was observed. The induction peaked at 1.0 × 10⁻⁸ M (4.01 ± 0.22 (SD)-fold, *n* = 4) and gradually fell at higher concentrations. The dose-response of well known less potent ecdysoid steroid, ecdysone, was also investigated (Fig. 3B). At 1.0 × 10⁻⁷ M, a 2.00 ± 0.24 (SD)-
fold \((n = 4)\) induction was observed and peaked at \(1.0 \times 10^{-6} \text{ M}\) (4.99 ± 0.32 (SD)-fold). This induction also fell at higher concentrations of ecdysone. However, although the inhibition of induction by higher doses of ecdysteroids was also observed at levels of acetylcholinesterase activities (Wing, 1988), the mechanism of this inhibition has not been elucidated.

Furthermore, the induction activities of dibenzoyl hydrazines (RH 5849 and RH 5992) which had been reported as a non-steroidal ecdysteroid agonists (Wing, 1988; Wing et al., 1988; Mikitani, 1996), were also investigated in the same system. RH 5849 showed a 3.55-fold \((n = 2)\) induction at \(2.0 \times 10^{-5} \text{ M}\) (Fig. 4). RH 5992, which has higher potential ecdysteroid agonist activities than RH 5849, achieved a 4.24-fold \((n = 2)\) induction at a 10-fold lower dose \((2.0 \times 10^{-6} \text{ M}, \text{ Fig. 4})\).

Luciferase induction of pDACH-E-LUC introduced Kc cells is approximately 10 times

Fig. 2. The time course of luciferase induction in pDACH-E-LUC introduced Kc cells by 20-hydroxyecdysone \((1.0 \times 10^{-4} \text{ M})\). Each luciferase activity corrected for protein concentration was divided by normalized luciferase activity of the control, and was defined as “Fold induction.” Each data point represents the mean ± SD \((n = 3)\).

Fig. 3. Dose-response curves for luciferase induction in pDACH-E-LUC introduced Kc cells by ecdysteroids 72 h after treatment. A, 20-hydroxyecdysone; B, ecdysone. Fold induction activities were obtained as described in the legend to Fig. 2. Each data point represents the mean ± SD \((n = 4)\).
more sensitive than that of pHSP27-LUC introduced Kc cells (Mikitani, 1995a) at a concentration of 20-hydroxyecdysone or ecdysone. This higher sensitivity level is also observed in Kc cell morphological changes of the process protrusion as a "neuroblast"-like morphology (Mikitani, 1995b). However, maximal induction is much smaller in the pDACHe-LUC plasmid-introduced Kc cells (4 to 5-fold) than it is in the pHSP27-LUC plasmid-introduced cells (80-fold). These differences between the two ecdysteroid responsive genes may suggest that the expression of the D. melanogaster Ace gene is not directly controlled by the ecdysteroid receptor complex (Ecr-Usp) liganded with ecdysteroid, as shown using the hsp27 gene ecdysteroid responsive element (Koelle et al., 1991; Yao et al., 1992, 1993; Thomas et al., 1993). Other ecdysteroid inducible transcription factors might function in Ace gene regulation. The time lag (more than 48 h after hormone treatment) of acetylcholinesterase induction, as well as the similarity in the dose-response of Kc cell morphological changes and Ace gene induction suggest that the Ace gene induction resulted from differentiation of Kc cells caused by ecdysteroids. In addition, no consensus ecdysteroid responsive elements, neither imperfect palindromic (Cherbas et al., 1991) nor direct repeats (D'Avino et al., 1995), exist in the Ace gene 5'-upstream 1.62 kb fragment used in the pDACHe-LUC plasmid. Only a part of the consensus sequence for the imperfect palindrome ecdysteroid responsive element, "TGAAC," was found from -1,192 bp to -1,188 bp upstream of the transcriptional start site of the Ace gene. Further studies of acetylcholinesterase induction, such as the deletion mutant analysis of the 1.62 kb 5'-upstream region of the pDACHe-LUC plasmid, and a northern blot analysis with protein synthesis inhibitors, are presently being carried out.

In the brain of prepupa and pupa of Tenebrio molitor, an induction of acetylcholinesterase activity was observed after the peak of the haemolymph ecdysteroid titer of each stage (LeNoir-Rousseaux et al., 1994). A region specific expression pattern of Ecr isoform was shown in D. melanogaster CNS at the larval-pupal stage (Truman et al., 1994). In vivo analysis of Ace gene expression in embryo or CNS regions of D. melanogaster using the transformation technique in combination with the Kc cell transfection assay described in this paper should elucidate the elements responsible for induction of this gene by ecdysteroid. In addition, the
pDAChE-LUC plasmid presented in this paper may be useful in an evaluation assay of ecldyosteroid agonist activity at the gene expression level.

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REFERENCES


