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Mode of Action of Nonsteroidal Ecdysone Agonists, Diacylhydrazine Analogs*

Chieka MINAKUCHI**

Department of Biology, University of Washington, Seattle, WA 98195–1800, USA

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The binding affinity of insect molting hormone agonists, *N-tert*-butyl-*N*,*N*′-diacylhydrazine (DAH) analogs, against the molting hormone receptor proteins (EcR/USP) was measured. For the lepidopteran *Chilo suppressalis*, the receptor-binding activity of DAH analogs correlated linearly with their molting hormonal activity (*in vitro*) and insecticidal activity (*in vivo*). The binding activity of DAH analogs of *C. suppressalis* EcR/USP was higher than that of the dipteran *Drosophila melanogaster* EcR/USP. The binding activity of EcR/USP changed little when USP was exchanged between *C. suppressalis* and *D. melanogaster*. These results suggest that the selective toxicity of DAH analogs toward Lepidoptera is caused by the difference in the structure of EcR, not by that of USP. © Pesticide Science Society of Japan

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INTRODUCTION

Insect molting is regulated via binding of the molting hormone (20-hydroxyecdysone: 20E) to its receptor protein, the heterodimer of ecdysone receptor (EcR) and ultraspiracle (USP). The 20E-EcR/USP complex binds to the ecdysone response element in the promoter of target genes and regulates their transcription. cDNAs of EcR and USP were first cloned for fruit flies *Drosophila melanogaster*, then for other insect species.

N-tert-butyl-*N*,*N*′-diacylhydrazine (DAH) and its analogs mimic 20E action, and have molting hormonal activity as well as insecticidal activity. Four potent analogs (tebufenozide, methoxyfenozide, halofenozide and chromafenozide) are currently on the market as safer insecticides with reduced mammalian toxicity. By analyzing X-ray crystal structure of ligand-EcR/USP complex, it was disclosed that the ligand-binding pockets of EcR overlap partially between a molting hormone analog (ponasterone A: PonA) and a DAH analog (BYI06830).

To date, the insecticidal activity of DAH analogs has been measured against various insect species. Interestingly, many analogs including three commercial compounds (tebufenozide, methoxyfenozide and chromafenozide) possess higher insecticidal activity against Lepidoptera than against insects of other taxonomic orders. However, molecular mechanism of the selective toxicity of DAH analogs is unclear.

In this study, we measured the binding activity of DAH analogs to the molting hormone receptor proteins (EcR/USP) of lepidopteran *Chilo suppressalis* in order to elucidate their mode of action. Moreover, we compared their binding activity to EcR/USP between *C. suppressalis* and dipteran *D. melanogaster* in order to discuss the mechanism of their selective toxicity among insect orders. We also measured their binding activity to hybrid-type EcR/USP between *C. suppressalis* and *D. melanogaster* to elucidate the structural factor that rules the selective toxicity.

MODE OF ACTION OF DIACYLHYDRAZINE ANALOGS IN LEPIDOPTERAN *CHILO SUPPRESSALIS*

cDNAs for EcR and USP from *C. suppressalis* (CsEcR, CsUSP) were cloned using RT-PCR techniques. These cDNAs encode 547 amino acids (CsEcR) and 410 amino acids (CsUSP), respectively. BLAST search showed that they are highly homologous to those reported from a number of other insect species, especially those of other lepidopteran species.

The full coding regions of CsEcR and CsUSP were cloned into plasmid vectors, and *in vitro* transcription/translation

* See Part II for the full Japanese article.

** To whom correspondence should be addressed.
E-mail: chieka@u.washington.edu

reaction was performed using rabbit reticulocyte lysate. The binding affinity of ligands was measured with the competition of a radiolabeled ecdysteroid analog, [^{3}H]PonA. It was shown that PonA specifically bound to CsEcR, but not to CsUSP. The binding affinity of PonA to CsEcR was remarkably enhanced in the presence of CsUSP, suggesting that the binding affinity to PonA is enhanced by the allosteric interaction between EcR and USP.

Previously, Nakagawa *et al*. evaluated the molting hormonal activity as the 50% effective concentration (EC_{50}) for the induction of chitin synthesis in cultured integuments of *C. suppressalis*, and the insecticidal activity as the 50% lethal dose (LD_{50}) by topical application to *C. suppressalis* larvae. They reported that, for a series of DAH analogs with various substituents on benzene rings, there was a linear correlation between the molting hormonal activity (pEC_{50}) and the insecticidal activity (pLD_{50}). In this study, the binding activity of a series of DAH analogs to the *in vitro* expressed CsEcR/CsUSP heterodimer was quantitatively evaluated as the reciprocal logarithm of the 50% inhibition concentration (pIC_{50}) for the competition with [^{3}H]PonA. It was shown that the binding activity of a series of DAH analogs was linearly correlated with the molting hormonal activity, as well as with the insecticidal activity. These results indicate that the receptor-binding affinity of DAH analogs determines the strength of their biological activities such as molting hormonal activity (*in vitro*) and insecticidal activity (*in vivo*) in *C. suppressalis*.

SELECTIVE TOXICITY OF DIACYLHYDRAZINE ANALOGS BETWEEN LEPIDOPTERA AND DIPTERA

As stated above, it is well known that the insecticidal activity of most DAH analogs is higher to Lepidoptera than to Diptera. It has been suggested that this selective toxicity would be attributed to the difference in their binding affinity to the molting hormone receptor (EcR/USP) between these two insect orders. To investigate this point further, the binding activity of a series of ecdysteroids and DAH analogs was compared between lepidopteran *C. suppressalis* and dipteran *D. melanogaster*. The binding activity of ecdysteroids such as PonA and 20E did not vary significantly between *C. suppressalis* and *D. melanogaster*. However, the binding activity of DAH analogs was significantly higher to *C. suppressalis* than *D. melanogaster*. Thus, structure-activity relationship (SAR) for the receptor-binding activity of DAH analogs was very different between *C. suppressalis* and *D. melanogaster*, whereas SAR for ecdysteroids was very similar. Therefore, it was indicated that the selective insecticidal toxicity of DAH analogs would be attributed to the difference in their binding affinity to EcR/USP between *C. suppressalis* and *D. melanogaster*.

It has been believed that EcR has an important role to interact with ligands directly, while USP is an essential heterodimerization partner of EcR. Recently, it was revealed by analyzing X-ray crystal structure of lepidopteran *Heliothis virescens* EcR/USP that both an ecdysteroid and a DAH analog interact with the ligand-binding pocket of EcR. Therefore, it would be reasonable to assume that the structure of EcR rules the biological activity of DAH analogs and thus causes their selective toxicity. However, it was unknown whether USPs from different insects have various potency to stabilize EcR/USP heterodimer or not. Since the primary structure of USPs is diverse among insects, it is possible that USP structure would contribute partly to the selective toxicity of DAH analogs among insect orders. In this study, the binding activity of ecdysteroid and DAH analogs to hybrid-type receptors (CsEcR/DmUSP and DmEcR/CsUSP) was compared with that to the wild-type receptors (CsEcR/CsUSP and DmEcR/DmUSP) to see if the ligand-binding affinity changes when USP is exchanged between *C. suppressalis* and *D. melanogaster*. The binding activity to ecdysteroids was consistent among CsEcR/DmUSP, DmEcR/CsUSP, CsEcR/CsUSP and DmEcR/DmUSP, indicating that these hybrid-type receptors as well as wild-type receptors have the function to bind to ecdysteroids with high affinity. On the other hand, the binding activity to DAH analogs was diverse among them, and importantly, CsEcR/CsUSP and CsEcR/DmUSP had significantly higher affinity to DAH analogs than DmEcR/DmUSP and DmEcR/CsUSP. For all the test compounds including both ecdysteroids and DAH analogs, the binding activity to CsEcR/DmUSP was in good correlation with that to CsEcR/CsUSP, while the binding activity to DmEcR/CsUSP was correlated with that to DmEcR/DmUSP. Thus, the binding activity of DAH analogs to EcR/USP changed little when USP was replaced to other insect's counterpart. From these results, it was concluded that the binding activity of DAH analogs to EcR/USP is ruled mainly by the structure of EcR, but not by that of USP. It was suggested that the difference in EcR structure among insect orders would probably be the main factor causing the selective toxicity of DAH analogs.