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Molecular studies on the ouabain binding site of the Na^+, K^+-ATPase in milkweed butterflies

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Summary. The Na^+, K^+-ATPase of the Monarch butterfly (*Danaus plexippus*) is insensitive to the inhibition by cardiac glycosides due to an amino acid replacement: histidine instead of asparagine at position 122 of the α-subunit representing the ouabain binding site. By PCR amplification of the DNA sequence of this site, a PCR product of 270 bp was obtained from DNA extracted from Danainae species (*Danaus plexippus*, *D.chrysippus*, *D.gillipus*, *D.philene*, *D.genutia*, *Tirumala hamata*, *Euploea* spp., *Parantica weiskei*, *P.melusine*), Sphingidae (*Daphnis nerii*) and mimics of milkweed butterflies (*Hypolimnas missipus*, *Limenitis archippus* and *L.arthemis*, Nymphalidae). Analysis of the nucleotide sequences revealed that the single point mutation in the ouabain binding domain (AAC-Asn for CAC-His) was present only in *Danaus plexippus*, but not in the other species investigated. Since these milkweed butterflies also store cardenolides, other structural modifications of the Na^+, K^+-ATPase may have occurred or other strategies of cardenolide tolerance have been developed.

Key words. Na^+, K^+-ATPase – cardiac glycosides – cardenolides – Lepidoptera – Danainae – Sphingidae – Nymphalidae

Introduction

Larvae of the Danainae butterfly family feed on milkweed plants, *e.g.* Asclepiadaceae rich in cardiac glycosides. These chemicals are being sequestered into the imago, which may become unpalatable for bird predators (Malcolm & Zalucki 1992). Since cardiac glycosides (cardenolides) specifically inhibit the Na^+, K^+-ATPase, these insects must have developed mechanisms to tolerate these dietary compounds. In the monarch butterfly (*Danaus plexippus*), a modification of the ouabain-binding site in the enzyme's α-subunit by replacing asparagine in position 122 for histidine was found to contribute to the cardenolide insensitivity of the butterfly (Holzinger *et al.* 1992; Holzinger & Wink 1996). However, in another danaid butterfly, *Danaus gillipus*, this point mutation has not occurred suggesting other mechanisms of cardenolide tolerance (Holzinger & Wink 1996).

In the present study, other milkweed (Danainae) and oleander feeding (Sphingidae) butterflies were analyzed to find out, whether they exhibit similar molecular changes at the ouabain-binding site of their Na^+, K^+-ATPase.

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Materials and methods

Butterflies from the following locations were used: *Danaus plexippus* from Virginia and Oklahoma, (USA), Bulolo (Papua New Guinea, PNG) and Negros (Philippines), *D. chrysippus* (Turkey, PNG, Philippines), *D. gillipus* (Florida, USA), *D. philene* (PNG), *D. genutia* (Khao Yai, Thailand); *Tirumala hamata*, *Euploea* spp., *Parantica weiskei*, *P. melusine*, and *Hypolimnas missipus* (Bulolo, PNG), *Limenitis archippus* and *L. arthemis* (Massachusettes, USA), and *Daphnis nerii* (Tunisia).

DNA isolation and PCR amplification of the ouabain-binding site

From the abdominal tissue of the dried insects, DNA was isolated using standard procedures, *e.g.* phenol/chloroform extraction and ethanol precipitation according to Maniatis *et al.* (1989). DNA was amplified by PCR (30 cycles) using the following oligonucleotide primers according to Holzinger *et al.* (1992): (A) 5′CTG TGG ATC(T) GGT(A) GC(A)G(T) ATT CTT(C) TGC TTT 3′ and (B) 5′ACC ATG TTC(T) TTG AAC(G) GAT TC C ATG ATC TT 3′. The PCR fragments were purified using Microcon 100 concentrators (Amicon, Witten Germany). Cycle sequencing was performed using the BigDye Terminator Cycle Sequencing Kit (Perkin Elmer, USA) according to the manufacturers recommendations. The annealing temperature was 55°C. Primer (B) was used in the sequencing reaction in a concentration of 10 pmol per reaction. Electrophoresis and detection of of the sequencing reaction products was done on the capillary electrophoresis system ABI Prism 310 Genetic Analyzer using POP (Performance Optimized Polymer) 6, with a capillary length of 47 cm and diameter of 50 μm.

Results

By using PCR primers to amplify the cardenolide binding site of the Na^+, K^+-ATPase, a single PCR product of 270 bp was obtained from the DNA isolated from the butterflies. Analysis of its nucelotide sequence was successful in all species investigated. The derived amino

Table 1 Partial nucleotide sequence of the ouabain binding site of the Na^+, K^+-ATPase of *Danaus plexippus*, leading to the replacement of the amino acid asparagine for histidine, compared to the sequence of another danaid species (*Danaus chrysippus*) and of one of its mimics (*Limenitis archippus*), where asparagine is conserved

	↓
Danaus plexippus	**GAA GAA CCC TCG GAT GAC CAC TTG TAT CTC GGA**
Danaus chrysippus	**GAA GAA CCC TCG GAT GAC AAC TTG TAT CTC GGA**
Limenitis archippus	**GAA GAA CCC GCG GAT GAC AAC TTG TAT CTT GGT**

Table 2 Derived amino acid sequences of the ouabain binding site (transmembrane domain H1 and H2, position 111 to 122) of several cardenolide-feeding butterflies (Danainae and Sphingidae) and of their mimics (Nymphalidae). Origin of the butterflies in brackets (PNG-Papua New Guinea)

Danainae:																				
	111									120		122								130
Danaus plexippus (USA, PNG, Philippines)	Gln	Ala	Ser	Thr	Val	Glu	Glu	Pro	Ser	Asp	Asp	**His**	Leu	Tyr	Leu	Gly	Ile	Val	Leu	Ala
Danaus chrysippus (PNG, Turkey)	–	–	–	–	–	–	–	–	–	–	–	Asn	–	–	–	–	–	–	–	–
Danaus gillipus (USA)	–	–	–	–	–	–	–	–	–	–	–	Asn	–	–	–	–	–	–	–	–
Danaus philene (PNG)	–	–	–	–	–	–	–	–	–	–	–	Asn	–	–	–	–	–	–	–	–
Danaus genutia (Thailand)	–	–	–	–	–	–	–	–	–	–	–	Asn	–	–	–	–	–	–	–	–
Tirumala hamata (PNG)	–	–	–	–	–	–	–	–	–	–	–	Asn	–	–	–	–	–	–	–	–
Euploea spp. (PNG)	–	–	–	–	–	–	–	–	–	–	–	Asn	–	–	–	–	–	–	–	–
Parantica weiskei (PNG)	–	–	–	–	–	–	–	–	–	–	–	Asn	–	–	–	–	–	–	–	–
Parantica melusine (PNG)	–	–	–	–	–	–	–	–	–	–	–	Asn	–	–	–	–	–	–	–	–
Sphingidae:																				
Daphnis nerii (Tunisia)	–	–	–	–	–	–	–	–	–	–	–	Asn	–	–	–	–	–	–	–	–
Mimics (Nymphalidae):																				
Hypolimnas missipus (PNG)	–	–	–	–	–	–	–	–	–	–	–	Asn	–	–	–	–	–	–	–	–
Limenitis archippus (USA)	–	–	–	–	–	–	–	–	Ala	–	–	Asn	–	–	–	–	–	–	–	–
Limenitis arthemis (USA)	–	–	–	–	–	–	–	–	Ala	–	–	Asn	–	–	–	–	–	–	–	–

acid sequence (position 111 to 130 of the α-subunit, the ouabain-binding site including the transmembrane domain, position 111 to 122; Table 2) confirmed the replacement of asparagine by histidine at position 122 for *Danaus plexippus* due to a single point mutation (AAC-Asn for CAC-His; Table 1), in specimens from the USA, Papua New Guinea and from the Philippines. However, other *Danaus* species, *e.g. Danaus chrysippus*, *D. gillipus*, *D. philene* and *D. genutia*, and other members of the danaid family, *e.g. Tirumala hamata*, *Euploea* spp., *Parantica weiskei*, *P. melusine*, lack this single point mutation and exhibit the same amino acid sequence as cardenolide sensitive butterflies, *e.g.* danaid mimics of the Nymphalidae family: *Hypolimnas missipus*, *Limenitis archippus* and *L. arthemis*. The same applies to *Daphnis nerii*; the larvae of this butterfly feed on *Nerium oleander*, which contains high levels of the cardenolide oleandrine. In the two *Limenitis* species, a serine residue in position 119 is replaced by an alanine residue.

Discussion

This study confirms the unique status of the monarch *Danaus plexippus* among several other members of the Danainae butterflies: it is the only species with an amino acid replacement in the ouabain-binding site of the Na^+, K^+-ATPase, which renders the enzyme insensitive to cardenolides (Holzinger & Wink 1996). Even the wide geographical distribution of this species, from USA over the Pacific to Papua New Guinea and the Philippines, had no negative inplications on its cardenolide insensitivity.

It is interesting to note that all other butterflies, where the larvae feed on cardenolide-containing plants (Ackery & Vane-Wright 1984), members of the Danainae and Sphingidae (*Daphnis nerii*), show identical amino acid sequences at the ouabain-binding site, carrying an asparagine residue at position 122, which may indicate that the enzyme of these butterflies is sensitive to cardenolides. A substitution at position 119 as seen in the *Limenitis* species (Nymphalidae) (alanine for serine) appears not to be significant. These butterfly species "mimic" only danaids and their larvae avoid cardenolide plants as food.

This particular mutation provides *Danaus plexippus* with the advantage to sequester cardenolides. Since the other danaids and *Daphnis nerii* also store these compounds in their tissues, other modifications at the Na^+, K^+-ATPase may have occurred, which prevent cardenolide binding, or these species have developed other strategies to tolerate the toxic cardenolides.

References

Ackery PR, Vane-Wright RI (1984) Milkweed Butterflies, Their Cladistics and Biology. British Museum (Natural History), London

Holzinger F, Frick C, Wink M (1992) Molecular basis for the insensitivity of the Monarch (*Danaus plexippus*) to cardiac glycosides. FEBS Letters 314:477–480

Holzinger F, Wink M (1996) Mediation of cardiac glycoside insensitivity in the monarch butterfly (*Danaus plexippus*): role of an amino acid substitution in the ouabain binding site of Na^+, K^+-ATPase. J Chem Ecol 22:1921–1937

Maniatis T, Fritsch EF, Sambrook J (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Lab. Press, Cold Spring Harbor

Malcolm, SB, Zalucki MP (eds) (1992) Biology and Conservation of the Monarch Butterfly. Los Angeles: Natural History Museum of Los Angeles County

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