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Molecular basis for the insensitivity of the Monarch (*Danaus plexippus*) to cardiac glycosides

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The Monarch (*Danaus plexippus*) sequesters cardiac glycosides for its chemical defence against predators. Larvae and adults of this butterfly are insensitive towards dietary cardiac glycosides, whereas other Lepidoptera, such as *Manduca sexta* and *Creatonotos transiens* are sensitive and intoxicated by ouabain. Ouabain inhibits the Na^+,K^+-ATPase by binding to its α-subunit. We have amplified and cloned the DNA sequence encoding the respective ouabain binding site. Instead of the amino acid asparagine at position 122 in ouabain-sensitive insects, the Monarch has a histidine in the putative ouabain binding site, which consists of about 12 amino acids. This change may explain the ouabain insensitivity.

Danaus plexippus; Ouabain insensitivity; Na^+,K^+-ATPase; Binding site; Nucleotide sequence

1. INTRODUCTION

Plants produce a wide variety of secondary metabolites, such as alkaloids, terpenoids and glycosides, many of which serve as antiherbivore or antimicrobial defence compounds [1–4]. However, a substantial number of mono- or oligophagous insect species have evolved which are highly adapted to the particular defence chemistry of their host plants. Often, they actively sequester the dietary allelochemicals and use them for their own defence against predators [1,5–10]. Examples include danaid and arctiid Lepidoptera, exploiting cardiac glycosides and pyrrolizidine alkaloids [5–10], or pyralid moths and aphids storing quinolizidine alkaloids [11].

Larvae of the Monarch butterfly (*Danaus plexippus*) and of other danaids feed on food plants rich in cardiac glycosides, especially taxa of the family of Asclepiadaceae [5-7]. Larvae sequester the dietary cardenolides and pass them via pupae to the adults which become unpalatable for predators such as Blue jays [5].

Cardiac glycosides inhibit the Na^+,K^+-ATPase in vertebrates and are thus toxic for most animals [12–14]. In insects, the Na^+,K^+-ATPase of neuronal tissue is especially sensitive to cardiac glycosides, such as ouabain [14]. A remarkable difference has been reported for the Monarch, whose Na^+,K^+-ATPase is far less sensitive than that of other phytophagous insects [14].

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In this study we discovered an amino acid exchange in the ouabain binding site of the Monarch Na^+,K^+-ATPase which may be responsible for its apparent insensitivity to cardiac glycosides.

2. MATERIALS AND METHODS

2.1. *Activity of Na^+,K^+-ATPase*

Heads of *D. plexippus*, *Manduca sexta* and *Creatonotos transiens* were homogenized in a Tris buffer [14] (100 mM NaCl, 10 mM $MgCl_2$, 50 mM Tris, pH 7.4). Reaction conditions for Na^+,K^+-ATPase: 200 μl buffer containing 50 μg protein, 1 mM ATP and respective amounts of ouabain were incubated for 20 min at 24°C. The reactions were terminated by adding 800 μl of a colorimetric test mixture (1.2 M H_2SO_4, 2% ascorbic acid, 0.5% ammonium molybdate in 2 ml H_2O) and evaluated photometrically at 820 nm. All assays were performed in triplicate and compared to untreated, ouabain-free controls.

2.2. *Isolation of DNA and PCR amplification of the ouabain binding site*

DNA was isolated from adult butterflies of *D. plexippus* or from larval guts of *M. sexta* and *C. transiens* using the CTAB method [15]. About 1.5 μg of DNA was amplified by *Taq* polymerase (30 cycles) and the oligonucleotide primers A: 5′ CTG TGG ATC(T) GGT(A) GCT(GC) ATT CT 3′; B: 5′ CTG TGG ATC(T) GGT(A) GC(A)G(T) ATT CTT(C;A) TGC TTT 3′; or C: 5′ ACC ATG TTC(T) TTG AAC(G) GAT TCC ATG ATC TT 3′ using standard conditions [16,17]. For *D. plexippus* and *M. sexta* primers A and C and for *C. transiens* primers B and C were employed. The PCR products were separated by agarose gel electrophoresis (1%) and purified by the Quiaex method (Diagen, Düsseldorf), followed by phosphorylation using polynucleotide kinase [18]. Aliquots were ligated with pUC21 (*HindII*) using T4 ligase. *E. coli* (JM 109) were transformed with the respective plasmids by electroporation (Gene pulser, Bio-Rad) and cultivated on LB medium with ampicillin [18]. Insert-containing clones were used for large scale plasmid isolation, which were sequenced according to the chain-termination method [16–18] using the sequenase protocol (USB). Autoradiograms were evaluated manually. For sequence comparisons, the EMBL data libraries were screened.

3. RESULTS AND DISCUSSION

3.1. *Ouabain insensitivity*

When injecting defined doses of ouabain into larvae of *D. plexippus*, we found no toxic effect up to a dose of 1,200 mg/kg, whereas an LD_{50} was reached in *M. sexta* at 20 mg/kg. The insensitivity of Na^+,K^+-ATPase from *D. plexippus* was apparent in vitro using homogenates from butterfly heads (Fig. 1). As expected, the enzyme of other Lepidoptera, which are ouabain sensitive and do not sequester cardiac glycosides [19], such as *M. sexta* and *C. transiens*, can be completely inhibited by 0.1 mM ouabain (Fig. 1).

3.2. *PCR amplification of the ouabain binding site*

How can we understand the sensitivity or insensitivity of Na^+,K^+-ATPase at the molecular level? The Na^+,K^+-ATPase consists of 2 subunits, a catalytic and ouabain-binding α-subunit with 1,016 amino acids, and a β-subunit with 302 amino acids [12,20]. The α-subunit

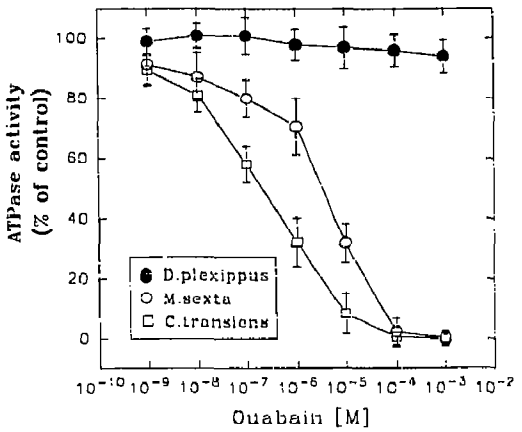

Fig. 1. Modulation of Na^+,K^+-ATPase by the cardiac glycoside ouabain. Enzyme preparations were obtained from ouabain-sensitive (*M. sexta*, *C. transiens*) and -insensitive insects (*D. plexippus*).

Table I

Partial nucleotide sequence of the H1 and H2 domain of the α-subunit of Na^+,K^+-ATPase of *D. plexippus*, *M. sexta*, *C. transiens* as compared to the known sequence of *Drosophila*.

```
                Primer A
                ----------
                1                   21                  41↓
Drosophila      CTGTGGATCG GTGCTATTCT CTGCTTTGTG GCCTATTCTA TCCAGGCCAG CACCAGCGAG
D.plexippus     CTGTGGATTG GAGAGATTCT ATGCTTTATT GCCTATGGTA TTCAGGCGAG TACTGTTGAA
M.sexta         CTGTGGATCG GTGCGATTCT TTGCTTTATT GCATATGGAA TCCAGGCGAG TACCGTAGAA
C.transiens     CTGTGGATTG GAGAGATTCT ATGCTTTATT GCCTATGGAA TTCAGGCTAG TACCGTAGAG
                ******** * * *  *****  ****** *  ** ***   * * ********  ** *  **

                61                  81                  101
Drosophila      GAGCCGGCCG ACGATAATTT GTATCTGGGT ATTGTACTTT CCGCTGTCGT CATCGTGACG
D.plexippus     GAACCCTCGG ACGACCACTT GTATCTCGGA ATCGTATTGG CGGCTGTCGT TATCGTGACT
M.sexta         GAACCCTCCG ATGATAACTT GTACCTTGGC ATTGTATTGG CGGCTGTCGT GATCGTTACG
C.transiens     GAACCAGCGG ATGACAATCT TTACCTCGGC ATCGTATTGG CAGCTGTCGT GATCGTTACG
                ** **  * * * * **  *  *  ** ** **  ** *** *   **********  ***** **

                121                 141                 161
Drosophila      GGCGTTTTCT CATACTATCA GGAATCGAAG AATTCTAAGA TCATGGAATC GTTCAAGAAC
D.plexippus     GGAATATTCT CTTACTACCA AGAAAGGAAG TCTTCCAAGA TCATGGAATC CTTCAAAAAC
M.sexta         GGTATATTCT CATACTACCA CGAAAGGAAG TCATCTAAGA TCATGGAATC GTTCAAAAAC
C.transiens     GGTATCTTCT CATACTATCA GGAAAGGAAG TCATCGAAGA TCATGGAATC GTTCAAAAAC
                **  * **** * ***** **  ***  ****    ** **** ********** ****** ***
                                                    ----------
                                                    Primer C

D.plexippus
INTRON          CTGGTAAGTT GTAGGCTCTT AATAACTTAA GTCATCTATT ATTTATTTTT GTGAGATGTT
                GAATATATTA CAACAACATT ATGAAACTC
M.sexta
INTRON          CAGGTAAGTT AATAAGCATG TCAATTACAT ATAGCGTTAT CAACGAAAAA ATATAATTTC
                GGAATAAAAT AATCCTCACA TAAGACAGCC TACCACGATG GACTCCTTTG GGTGAATATT
                CCCATATCAA ATAAAATTCT TTTACTTAAT TTATACCATC ATCACTTAAC AAACGAAAAA
                CATCAATGAA TTGGCTGGCT GGTCTGATCA TCAGTATAAA CTGAAGTTTT TGTCTTC
C.transiens
INTRON          CAGGTAAGTA ACGCAACAAA TACAATATAC TCAATAGCAA GTATACGACT TATAATTAAT
                ATGGCAAATA CTCCTC
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The ouabain binding site (position 43–79) is printed in bold. Introns were inserted between nucleotide positions 42 and 43, marked by an arrow.

Table II

Derived amino acid sequences of the ouabain binding site of ouabain-sensitive and insensitive organisms [12,13]

Sensitive enzymes												
	111				115					120		122
Human	Gln	Ala	Ala	Thr	Glu	Glu	Glu	Pro	Gln	Asn	Asp	Asn
Sheep	Gln	Ala	Ala	Thr	Glu	Glu	Glu	Pro	Gln	Asn	Asp	Asn
Drosophila	Gln	Ala	Ser	Thr	Ser	Glu	Glu	Pro	Ala	Asp	Asp	Asn
M. sexta	Gln	Ala	Ser	Thr	Val	Glu	Glu	Pro	Ser	Asp	Asp	Asn
C. transiens	Gln	Ala	Ser	Thr	Val	Glu	Glu	Pro	Ala	Asp	Asp	Asn
Insensitive enzymes												
	111				115					120		122
Rat	Arg	Ser	Ala	Thr	Glu	Glu	Glu	Pro	Pro	Asn	Asp	Asp
Mutant B*	Arg	Ala	Ala	Thr	Glu	Glu	Glu	Pro	Gln	Asn	Asp	**Asp**
D. plexippus	Gln	Ala	Ser	Thr	Val	Glu	Glu	Pro	Ser	Asp	Asp	**His**

*Sheep α1 cDNA, mutant B [13].

shows 8 transmembrane domains, and the main ouabain binding site is probably positioned in the extracellular loop between transmembrane domains H1 and H2 (amino acid position 111–122) [12]. This ouabain binding site covers 36 nucleotides. In rats, a ouabain-insensitive enzyme has been reported in which the substitution of arginine for a glutamine at position 111, and aspartic acid for an asparagine at position 122, is responsible, as compared to sensitive Na^+,K^+-ATPases from man or sheep [12]. Recently, evidence was presented that, in addition, also the first transmembrane segment of Na^+,K^+-ATPase confers ouabain resistance in MDCK canine cells [23].

Using the sequence information available [12,20], oligonucleotide primers were constructed to amplify the H1–H2 loop of Na^+,K^+-ATPase from sensitive and non-sensitive insects by PCR (Fig. 2, Table I). Nucleotide sequences obtained from *D. plexippus*, *M. sexta* and *C. transiens* showed a high homology to those of the α-subunits from other organisms, especially to that from *Drosophila* (Table I). Introns of different length were found between amino acid positions 109 and 110, i.e. just adjacent to the ouabain binding site (Table I).

Comparing the nucleotide sequences and the derived amino acid sequences of the ouabain binding site (Tables I and II, Fig. 2), 9 of the 12 amino acids of the insects studied are identical. Changes at positions 5 and 9 are probably not significant [12]. However, position 12 is critical [12] and shows a histidine in *D. plexippus*, whereas ouabain-sensitive insects and vertebrates carry an asparagine instead. Since histidine changes the receptor properties to a high degree, we assume that this target site modification in Monarchs may lead to a Na^+,K^+-ATPase which no longer binds ouabain. It cannot be ruled out that other mutations, e.g. in the first transmembrane segment [23] or in other extracellular loop regions, may be involved additionally in ouabain insensitivity.

Insensitivity towards cardiac glycosides is of ecological importance for the Monarch. Only due to this prop-

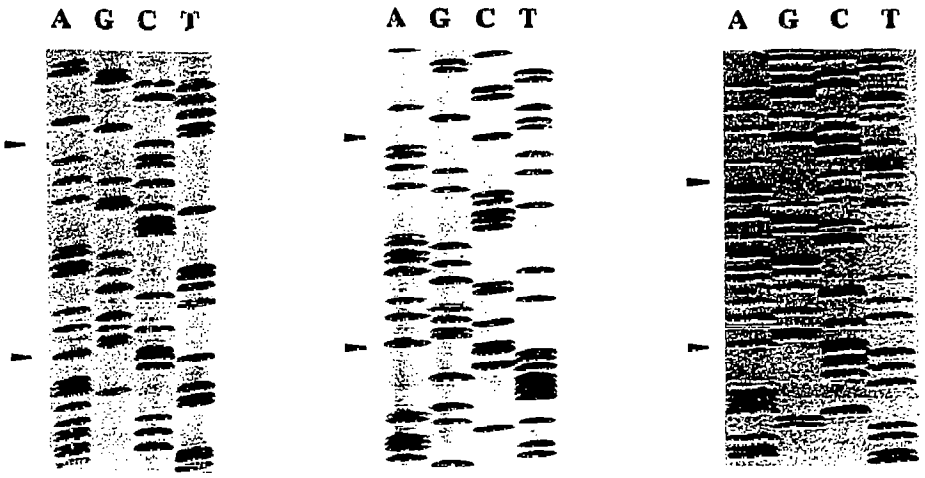

Fig. 2. Autoradiogram of the nucleotide sequence of the ouabain binding site (between arrows) in *D. plexippus*, *M. sexta* and *C. transiens*.

erty are these insects able to exploit the defence chemistry of their host plants for their own defence. It would be interesting to know whether other insects that store cardiac glycosides [21,22], such as *Oncopeltus fasciatus*, *Aphis nerii* or *Syntomeida epilais*, have followed a similar strategy during evolution.

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