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SHORT COMMUNICATION

Target-site sensitivity in a specialized herbivore towards major toxic compounds of its host plant: the Na^+K^+-ATPase of the oleander hawk moth (*Daphnis nerii*) is highly susceptible to cardenolides

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Abstract The caterpillars of the oleander hawk moth, *Daphnis nerii* (Linnaeus, 1758) (Lepidoptera: Sphingidae) feed primarily on oleander (*Nerium oleander*). This plant is rich in cardenolides, which specifically inhibit the Na^+K^+-ATPase. Since some insects feeding on cardenolide plants possess cardenolide-resistant Na^+K^+-ATPases, we tested whether *D. nerii* also possesses this strategy for circumventing cardenolide toxicity. To do so, we established a physiological assay, which allowed direct measurement of Na^+K^+-ATPase cardenolide sensitivity. Using *Schistocerca gregaria*, as a cardenolide-sensitive reference species, we showed that *D. nerii* Na^+K^+-ATPase was extremely sensitive to the cardenolide ouabain. Surprisingly, its sensitivity is even higher than that of the cardenolide-sensitive generalist, *S. gregaria*. The presence or absence of cardenolides in the diet of *D. nerii* did not influence the enzyme's cardenolide sensitivity, indicating that target-site insensitivity is not inducible in this species. However, despite the sensitivity of their Na^+K^+-ATPase, caterpillars of *D. nerii* quickly recovered from an injection of an excessive amount of ouabain into their haemocoel. We conclude that *D. nerii* possesses adaptations, which enable it to feed on a cardenolide-rich diet other than that previously described in cardenolide specialized insects, and discuss other potential resistance mechanisms.

Keywords *Daphnis nerii* · *Nerium oleander* · Cardenolides · Ouabain · Na^+K^+-ATPase · Adaptation · *Schistocerca gregaria*

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Introduction

Cardenolides and bufadienolides are also referred to as cardiac glycosides (Falbe and Regitz 1995) due to their therapeutic usage in the treatment of heart disease. These compounds occur in 12 different plant families (Luckner and Wichtl 2000), most prominently in the Apocynaceae, e.g. in oleander (*Nerium oleander*), a widely used ornamental plant toxic to humans and other animals (Frohne and Pfänder 2004). Cardenolides, the principal toxic components of *N. oleander*, possess a highly specific mode of action: they bind to and inhibit the Na^+K^+-ATPase (Schatzmann 1953), a ubiquitous transmembrane enzyme in the cells of all higher eukaryotes that is involved in essential physiological processes, such as nervous function and excretion (Lingrel 1992). Due to their ubiquitous target site, these compounds are expected to cause toxic effects in virtually all animals. Despite the toxic potential of these substances, there are several herbivorous insects of different orders, which feed on cardenolide plants.

In this paper, we investigated potential Na^+K^+-ATPase-cardenolide resistance in *Daphnis nerii* (Linnaeus, 1758), the oleander hawk moth (Lepidoptera: Sphingidae). The caterpillars of this species use *N. oleander* as their major host plant (Pittaway 1993) and are clearly able to cope with the toxic compounds, small amounts of which are also present in the larval body (Abe et al. 1996). However, it remains unknown how the caterpillars avoid intoxication by the cardenolides in their diet.

One possible adaptation to decrease cardenolide toxicity could be the evolution of a cardenolide-resistant Na^+K^+-ATPase similar to that found in the monarch butterfly (*Danaus plexippus*). Monarch larvae feed on toxic apocynacean plants and store high amounts of cardenolides in their body (Brower et al. 1982; Parsons

1965). The relative insensitivity of monarch Na^+K^+-ATPase to ouabain, a cardiac glycoside that occurs naturally in the apocynacean genera *Acokanthera* and *Strophanthus* in east Africa (Jäger et al. 1965) and which is widely used as a commercially available inhibitor of cellular Na^+K^+-ATPase activity, was first demonstrated by Vaughan and Jungreis (1977) using an enzymological assay. Later analyses showed that this insensitivity was due to an amino acid substitution (Asn122His) in the first extracellular loop of the protein, which is involved in ouabain binding (Holzinger et al. 1992). Convergently evolved cardenolide-insensitive Na^+K^+-ATPases were also detected in *Oncopeltus fasciatus* (Heteroptera: Lygaeidae), *Poekilocerus bufonius* (Caelifera: Acrididae), *Chrysochus auratus* and *C. cobaltinus* (Chrysomelidae) (Al-Robai 1993; Moore and Scudder 1986; Labeyrie and Dobler 2004).

However, not all cardenolide adapted species investigated so far contained a similar substitution in the first extracellular loop of their Na^+K^+-ATPase protein sequence and this was also true for *D. nerii* (Holzinger and Wink 1996; Mebs et al. 2000). Resistance could also be achieved by other alterations in the Na^+K^+-ATPase, since other regions of this protein are involved in the binding of ouabain (Croyle et al. 1997; Holzinger et al. 1992; Mebs et al. 2000; Qiu et al. 2005). Other forms of resistance are also hypothetically possible, such as guts impermeable to cardenolides or the production of cardenolide-degrading enzymes.

The aim of this study was to test whether the oleander specialist *D. nerii* also possesses an insensitive Na^+K^+-ATPase. To test this hypothesis, we performed a physiological assay, which allowed us to directly investigate the ouabain sensitivity of the *D. nerii* Na^+K^+-ATPase. Since caterpillars are the developmental stage, which is directly confronted with the cardenolides of the host plant, we used nervous tissue of caterpillars to extract the enzyme.

Additionally, we tested whether the sensitivity of the enzyme was altered by the presence of cardenolides in the diet by comparing the enzyme of *D. nerii* larvae raised on *N. oleander* with the enzyme of *D. nerii* caterpillars raised on *Vinca major* (Apocynaceae), a plant devoid of cardenolides. For comparison, and as a positive control of our method, we also assayed the Na^+K^+-ATPase of *Schistocerca gregaria* (Caelifera, Acrididae), an insect that is known to possess a Na^+K^+-ATPase sensitive towards ouabain (Moore and Scudder 1986). Finally, to test whether *D. nerii* caterpillars could tolerate high amounts of ouabain within the body cavity, we injected the larvae with a sufficient dose of the toxin such that the haemolymph level corresponded to a concentration causing total in vitro inhibition of the enzyme.

Materials and methods

Na^+K^+-ATPase-assays

We used *D. nerii* of different genetic backgrounds: some specimens originated from a cross between a strain from Thailand and European individuals, whilst others were derived from different European strains. The caterpillars were raised from eggs at room temperature and ambient light (one replicate), or in a climatic chamber at 27°C and constant light (six replicates), and fed either with fresh leaves of potted *N. oleander* plants or with cuttings of *V. major* collected from surrounding parks and gardens. To extract the Na^+K^+-ATPase, we used four (one replicate) or five (six replicates) fully grown last instar caterpillars. The caterpillars were anaesthetized on ice and decapitated. The nerve cord and the brain were dissected on ice, cleaned from adherent tissue and rinsed with deionized water.

The Na^+K^+-ATPase of *S. gregaria* was extracted from dissected brains and thoracic ganglia of nine adult locusts (three per replicate). The nervous tissues were pooled or singly homogenized on ice in a glass homogenizer with 1 ml deionized water using a motor-driven Teflon pestle (3 min at 800 rpm). Extracts of pooled nervous tissues were diluted with deionized water to 1 ml water per nervous tissue and aliquoted (1 ml) in Eppendorf tubes. Lyophilized extracts were stored at −80°C for a maximum of 9 weeks before use.

Prior to use, the lyophilisates were reconstituted with 100 µl of deionized water by vortex stirring and sonication in an ultrasonic bath. Pooled extracts were centrifuged at 1,000*g* at 4°C for 10 min to remove undissolved material. The supernatant was diluted with deionized water to reach a sufficient volume and the protein content was determined using the Bradford assay (Bradford 1976). Reaction conditions were similar to those described in Moore and Scudder (1986). Three different buffer conditions (I–III) were used to determine the degree to which the Na^+K^+-ATPase can be inhibited by ouabain and the Na^+K^+-ATPase activity relative to other ATPases: (I) 100 mM NaCl, 20 mM KCl, 4 mM $MgCl_2$, 50 mM imidazole (non-inhibited control); (II) 100 mM NaCl, 20 mM KCl, 4 mM $MgCl_2$, 50 mM imidazole with 10^{-3} to 10^{-8} M ouabain (Fluka) (determination of ouabain sensitivity); (III) 100 mM NaCl, 4 mM $MgCl_2$, 50 mM imidazole (determination of the activity of other ATPases: Na^+K^+-ATPase was not active since K^+ was lacking). Reactions were performed in Eppendorf tubes and consisted of the respective buffer (pH 7.4), Tris–ATP (Sigma-Aldrich) with a final concentration of 2.5 mM and ouabain solution or water, respectively. The reaction tubes were preincubated in a water bath at 37°C for several minutes and the reactions started by adding the tissue extract, which brought the

volume to 500 µl. After 20 min of incubation, reactions were stopped by the addition of 250 µl of 30% trichloroacetic acid (TCA) and centrifuged at 12,000*g* for 5 min to precipitate denatured proteins. ATPase activity was determined by quantifying released phosphate in a microplate reader (Biorad Model 680) at 655 nm using the photometric method described by Taussky and Shorr (1953). The concentration of endogenous phosphate in the samples and the amount of phosphate originating from non-enzymatic hydrolysis of ATP were quantified in control tubes containing buffer, water and either no ATP or no enzyme solution. Alongside with each series of samples, we ran a phosphate calibration curve using a standard series of 0.2–1.2 mM KH_2PO_4 in water mixed 1:2 with 30% TCA as in the reactions. A solution of 30% TCA in water (1:2) was used as a blank for all samples.

For further analysis, the enzyme activity was calculated as the percentage of control after subtracting the activity of other ATPases. To calculate the ouabain doses causing 50% inhibition (IC_{50}), the averaged curves of each species or treatment were fitted with the solver tool of Microsoft Excel 2003 using a five-parameter logistic function.

Ouabain injections

Daphnis nerii caterpillars were raised on *V. major* at 27°C (13/11-h light/dark cycle) to the penultimate instar. Larvae ($n = 10$) were weighed and injected with 10 µl of 10^{-2} M ouabain in 0.9% NaCl into the posterior dorsal vessel. Control larvae ($n = 10$) were injected with 0.9% NaCl only. Afterwards, individualized caterpillars were returned to the 27°C chamber and checked for toxic effects.

Results and discussion

The Na^+K^+-ATPase of the nervous tissue of *D. nerii* caterpillars was highly sensitive to the cardiac glycoside ouabain (Fig. 1). The Na^+K^+-ATPase of *D. nerii* was even more strongly inhibited by ouabain than the Na^+K^+-ATPase of *S. gregaria* that is well known to be sensitive to cardenolides. Whilst the overall activity of the Na^+K^+-ATPase was very similar in both species, the ouabain dose causing 50% inhibition of the enzyme was more than twice as high in *S. gregaria* compared to *D. nerii* (Table 1). Because the pharmacodynamics of all cardiac glycosides is regarded to be uniform (Luckner and Wichtl 2000), we assume that the Na^+K^+-ATPase of *D. nerii* can be inhibited by any cardenolide. Therefore, *D. nerii* possesses a target site highly vulnerable towards cardenolides, the major toxic compounds of its host plant.

There was no noticeable difference in the enzyme's cardenolide sensitivity on comparing caterpillars raised on cardenolide-rich oleander leaves (Fig. 1) and caterpillars fed with *V. major*, which is devoid of cardenolides, and the doses of ouabain that caused 50% inhibition were similar (Table 1). Thus, there is no indication that the occurrence of cardenolides in the diet alters the cardenolide sensitivity of the Na^+K^+-ATPase (e.g. by the expression of different isoforms of the enzyme).

Daphnis nerii was previously reported not to take up cardenolides (Rothschild et al. 1970). However, Abe et al. (1996) showed that *D. nerii* caterpillars do contain oleander cardenolides in their body. These authors observed different HPLC profiles on comparing the cardenolides derived from whole larvae with those derived from the faeces. Although they did not remove the guts of the caterpillars before extraction, contamination by plant material in the gut likely played a minor role in Abe et al.'s (1996) findings, because they used caterpillars immediately before pupation where empty guts could be expected. This indicates strongly that at least part of the detected cardenolides

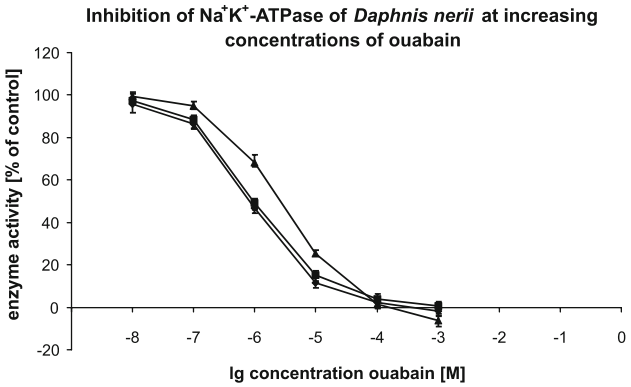

Fig. 1 Inhibition of the Na^+K^+-ATPase of *D. nerii* and *S. gregaria* by ouabain. *Filled squares* enzyme from caterpillars raised on *V. major*, *filled diamonds* enzyme from caterpillars raised on *N. oleander*, *filled triangles* enzyme from nervous tissue of *S. gregaria*. Each curve is the average of four (*D. nerii* from *N. oleander*) or three (*D. nerii* from *V. major* and *S. gregaria*) replicates; *bars* indicate minimum and maximum values. To estimate the contribution of Na^+K^+-ATPase to total P_i-release, KCl was omitted in separate control tubes. The amount of P_i measured under these conditions was subtracted from the overall release. The *negative values* in the *S. gregaria* curve might be due to a small remainder of Na^+K^+-ATPase activity potentially due to contamination with K^+ present in the extract

Table 1 In vitro enzyme activities and ouabain doses causing 50% inhibition of the Na^+K^+-ATPase (IC_{50})

	Na^+K^+-ATPase activity (nmol P_i/mg protein min) ± SD	IC_{50}
D. nerii from *N. oleander*	121.05 ± 18.86	9.1×10^{-07}
D. nerii from *V. major*	100.89 ± 15.29	1.04×10^{-06}
S. gregaria	82.02 ± 17.18	2.81×10^{-06}

originate from the larval body. Based on their results, the authors estimated the amount of bioactive cardenolides to be up to 150–200 µg per larva.

This amount of cardenolides should be sufficient to cause a drastic inhibition of the enzyme, yet in vivo the larvae obviously cope with these concentrations. In addition, our injection experiment also showed that *D. nerii* caterpillars can tolerate a high amount of ouabain within the body cavity. Each caterpillar (mean weight ~400 mg) received 10 µl 10^{-2} M of ouabain solution bringing the haemolymph to a level of $\sim 10^{-4}$ M at minimum, a concentration causing almost total inhibition in vitro (Fig. 1). All caterpillars injected with ouabain showed slight signs of toxic effect: they reacted lethargically when turned on their side and sometimes stayed in that position. Whilst healthy caterpillars adhere tightly to surfaces, these injected caterpillars did not. Caterpillars that had received only saline were much more vital and displayed normal behaviour. At the end, however, all caterpillars recovered and resumed feeding. These findings potentially parallel the results of Vaughan and Jungreis (1977). Although they did not observe toxic effects in *Manduca sexta* caterpillars after the injection of a similar amount of ouabain, the authors showed that the bulk of the toxin was excreted, whilst metabolic degradation could not be detected. The initial toxic effect in the *D. nerii* larvae and the slow recovery might correspond with the excretion of ouabain in these caterpillars.

In contrast to the situation in the monarch, there is no report that *D. nerii* derives chemical protection by the sequestration of cardenolides. Brower et al. (1982) reported that the cardenolide content of adult monarchs averaged 616 µg. The cardenolide content of an adult *D. plexippus* is, therefore, at least three times higher than the cardenolide content of a *D. nerii* larva. In addition, the total weight of an adult monarch (~0.6 g, own data, $n = 6$) is only about one-tenth of the weight of a *D. nerii* larva (Abe et al. 1996: 5.56 g), making the concentration of cardenolides in *D. nerii* caterpillars only about one-thirtieth of that observed in *D. plexippus.* Moreover, adynerin, the dominant cardiac glycoside in the larvae detected by Abe et al. (1996) is reported as not eliciting cardiotonic activity (Imai et al. 1965).

The relatively low concentration of cardenolides and the camouflaged habit of the caterpillar (Rothschild 1985), as well as of the moth, suggest a cryptic, rather than an aposematic, lifestyle. Our finding that the Na^+K^+-ATPase of *D. nerii* is highly sensitive towards ouabain provides indirect evidence that this species possesses mechanisms of resistance other than a modification of the Na^+K^+-ATPase. The relatively low cardenolide content of the larvae suggests that *D. nerii* might absorb only an 'unavoidable' part of dietary cardenolides whilst excluding the main part of the toxins, which pass through the gut. The predominance of adynerin in the caterpillars, a minor compound in the plant (Tschesche and Bohle 1938), may indicate quantitative differences in the absorption of different cardenolides. This might be due to differences in the physical properties of the molecules. Generally, more polar cardenolides should be easier to exclude than less polar ones, due to the passive membrane permeation of lipophilic compounds. Mechanisms that prevent absorption of cardenolides are plausible, because they are reported to exist even in species not adapted to cardenolides: tracer feeding experiments demonstrated that neither of the generalists, *S. gregaria* and *Periplaneta americana*, takes up radioactively labelled cardenolides via their guts (Scudder and Meredith 1982) nor does the leaf beetle, *Chrysochus asclepiadeus*, a close relative of cardenolide sequestering species that itself is not naturally exposed to cardenolides (Dobler 2004). These observations cannot, however, reveal whether the uptake of cardenolides is prevented or whether they are immediately removed from the haemolymph by an efficient excretion mechanism as observed in *Drosophila melanogaster* (Torrie et al. 2004).

The occurrence of cardenolide barriers in guts of generalist insects possibly indicates that cardenolide-rich host plants can also be used by non-specialized insects. Such mechanisms might explain how polyphagous insects avoid intoxication by cardenolides in their diets (e.g. *Gymnoscelis rufifasciata* and *Eupithecia* spp. on *Digitalis purpurea*). However, there are also reports of polyphagous lepidopteran species, which show toxic effects due to the ingestion of cardenolides. Dussourd and Hoyle (2000) show clearly that caterpillars of several generalistic noctuid moths suffer spasms after the ingestion of cardenolide solutions or latex of *Asclepias* species, which contains cardenolides. Furthermore, Karowe and Golston (2006) showed that the cardenolide digitoxin at lower doses deterred the caterpillars of *Lymantria dispar* (Lepidoptera: Lymantriidae) from eating and caused toxic effects at higher doses. These results imply that neither an impermeable gut nor the ability to tolerate cardenolides within the haemocoel is a general feature of lepidopteran larvae. Based on our observation that *D. nerii* caterpillars can tolerate excessive amounts of ouabain within their body cavity, we postulate that the target site of cardenolides, the Na^+K^+-ATPase, is insulated from the toxins. Na^+K^+-ATPase is abundant in the nervous tissue of insects (Emery et al. 1998). Moreover, immunohistochemical investigations indicate that the Na^+K^+-ATPase in the nervous tissue of caterpillars is expressed at a disproportionately high level and is not detectable elsewhere (G. Petschenka, unpublished data). For this reason the perineurium, which ensheathes the nervous system, could play an important role in protecting the Na^+K^+-ATPase (see Lebovitz et al.

1989). Further experiments focussing on the absorption of cardenolides via the gut, on cardenolide excretion and on the physiological properties of the "blood–brain barrier", of *D. nerii* caterpillars might enhance our understanding of the multilayer phenomenon of cardenolide resistance.

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