Different combinations of insect Na,K-ATPase α - and β -subunit paralogs enable fine tuning of toxin resistance and enzyme kinetics

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Summary statement

Activity and cardiac glycoside resistance of the *Oncopeltus fasciatus* Na,K-ATPase is determined by amino acid substitutions in the α -subunit binding pocket, the α/β -subunit combination and the cardiac glycoside identity.

Abstract

Cardiac glycosides are known to fatally inhibit the Na,K-ATPase throughout the animal kingdom. Several animals, however, evolved target-site insensitivity by substitution in the otherwise highly conserved cardiac glycoside binding pocket located on the Na,K-ATPase α -subunit. The minimal functional enzyme consists of an α - and a β -subunit, considered mainly as a chaperone responsible for correct folding and membrane integration. We here analyze resistance to cardiac glycosides and kinetic properties of different Na,K-ATPase α - β -combinations of the large milkweed bug, *Oncopeltus fasciatus*. These insects have adapted to high concentrations of cardiac glycosides in their food plants via several rounds of ATPase $\alpha 1$ gene duplications followed by subfunctionalization of the enzymes. To investigate their characteristics we expressed nine combinations of Na,K-ATPase α/β -subunits (three each) in Sf9 cells and tested them with two structurally distinct cardiac glycosides, calotropin, a host plant compound, and ouabain, a commonly used toxin. Differences in the number and identity of amino acid substitutions in the cardiac glycoside binding site resulted in large differences in activity and toxin resistance of the three α -

subunits. The enzymes' kinetics were also influenced by the β -subunits leading to increased activities ($\alpha\beta 3$) or altered resistances. Calotropin proved to be a much more potent inhibitor than ouabain. Our results show that the possession of multiple paralogs enables adaptation to plant toxins and mitigates pleiotropic effects by a compromise between ion pumping activity and resistance.

Introduction

The transmembrane Na,K-ATPase, is ubiquitously expressed in animal cells and is essential for maintaining membrane potentials, controlling cell volume and the overall functionality of cells (Horisberger, 2004). The functional enzyme consists of two subunits, the catalytic α -subunit and an associated β -subunit serving as chaperone for correct folding and membrane integration (Geering, 2001). In vertebrates several paralogs are present for both genes and the enzyme's characteristics differ depending on the specific combination of α - and β -subunits (Blanco, 2005; Blanco and Mercer, 1998). An additional modulatory and tissue specific γ -subunit is present in vertebrate tissues (Geering, 2005), but has not been detected in invertebrates. Much less is known about the importance of different α - β -subunit combinations in invertebrates (Okamura et al., 2002). Only in *Drosophila* the tissue distribution and functional roles of some of these combinations have been explored to some extent (Baumann et al., 2010; Paul et al., 2007; Yasuhara et al., 2000). We here explore the functional role of different Na,K-ATPase complexes in the large milkweed bug, *Oncopeltus fasciatus*, that have undergone three rounds of duplications of the Na,K-ATPase (Yang et al., 2019; Zhen et al., 2012) and display high insensitivity against cardiac

glycosides (Bramer et al., 2015; Moore and Scudder, 1986), which are universal inhibitors of the Na,K-ATPase (Horisberger, 2004).

Inhibition of the Na,K-ATPase by cardiac glycosides, such as the cardenolides ouabain and calotropin (Fig. 1), depends on a specific binding site located on the extracellular side of the catalytic α -subunit. Upon binding of cardiac glycosides the enzyme is arrested in the phosphorylated state and ion pumping is disrupted (Horisberger, 2004). Inhibition of the Na,K-ATPase is harmful and can be lethal to almost all animals. There are, however, a few notable exceptions to this: various mammals, frogs and reptiles prey unharmed on cardiac glycoside-containing toads and a number of phytophagous insects feed - often exclusively - on cardiac glycoside-containing host plants (reviewed in Agrawal et al., 2012; Malcolm, 1991). Most of these species avoid the toxic effect of cardiac glycosides and exhibit target site insensitivity, by drastically lowering binding affinity of cardiac glycosides to their Na,K-ATPase α -subunit (reviewed in Petschenka et al., 2017; Ujvari et al., 2015). Strikingly, identical amino acid substitutions at homologous positions convergently evolved in various, distantly related insect groups and even resemble the substitutions observed in cardiac glycoside-resistant vertebrates (Dobler et al., 2012; Mohammadi et al., 2016; Ujvari et al., 2015; Zhen et al., 2012).

While insect species usually express a single gene copy of the Na,K-ATPase α -subunit (ATP α 1) throughout their body, this gene underwent one to three rounds of gene duplications in several cardiac glycoside-exposed species (Dobler et al., 2019; Petschenka et al., 2017; Yang et al., 2019; Zhen et al., 2012). These duplicated ATP α 1 copies differ in the number and identity of presumably resistance-causing amino acid substitutions and

show tissue specific expression patterns (Dobler et al., 2019; Lohr et al., 2017; Yang et al., 2019; Zhen et al., 2012).

The large milkweed bug *Oncopeltus fasciatus* (Hemiptera, Lygaeidae) possesses four ATP α 1 paralogs (A, B, C, D) that differ in the number and identity of amino acid substitutions (Yang et al., 2019; Zhen et al., 2012). We previously introduced substitutions observed in these paralogs at four usually highly conserved amino acid sites (Q111, N122, F786 and T797) known to be important for high affinity to cardiac glycosides (Croyle et al., 1997; Laursen et al., 2015; Palasis et al., 1996; Price and Lingrel, 1988) into the sensitive ATP α 1 gene of *Drosophila melanogaster* (Dalla and Dobler, 2016; Dalla et al., 2017). The N122H (as observed in ATP α 1D of *O. fasciatus*), N122H+T797S (as observed in ATP α 1C), Q111T+N122H+F786N (as in ATP α 1B) or Q111T+N122H+F786N+T797A (as in ATP α 1A) substitutions yielded proteins with stepwise increasing resistance to the cardenolide ouabain. At the same time, enzyme activity decreased with increasing numbers of substitutions (Dalla and Dobler, 2016).

Additional untested substitutions are present in the *O. fasciatus* ATP α 1 paralogs and these might influence the characteristics of the enzymes, potentially further increasing resistance but possibly also serving as compensatory mutations mitigating deleterious intra-molecular epistatic effects of previously characterized mutations (Karageorgi et al., 2019; Taverner et al., 2019). In addition, it has been shown that the β -subunit of the Na,K-ATPase not only acts as a chaperone, guiding the heterodimeric enzyme to the cell membrane, but also influences the behavior of the Na,K-ATPase including its sensitivity to ouabain (Blanco, 2005; Dalla et al., 2017; Geering, 2001).

Although ouabain is a commonly used cardiac glycoside in studies of the Na,K-ATPase, it does not occur in host plants of *O. fasciatus*. The *Asclepias* species (Apocynaceae) that the bugs feed on typically contain different types of cardenolides characterized by a *trans-trans-cis* conformation of the steroidal skeleton and a single sugar moiety attached by a cyclic bridge as in the abundant calotropin (Malcolm, 1991) (Fig. 1). The most biologically relevant test of the bugs' resistance against cardiac glycosides should thus involve a plant-derived compound like calotropin. To address these issues, we identified and heterologously expressed nine possible *O. fasciatus* Na,K-ATPase α/β combinations and characterized their activities and sensitivities to ouabain and calotropin.

Since activity and resistance of the Na,K-ATPase is determined by the number and identity of amino acid substitutions in the cardenolide binding site located in the α -subunit, (1) we hypothesized that α -subunit paralogs – associated with different β -subunits – differ significantly in their activities and insensitivities to the two cardenolides we tested. (2) We further hypothesized that the different β -subunits modulate the Na,K-ATPase α -subunit paralog behavior influencing their response to the cardenolides. Additionally, although ouabain and calotropin both seem to be strong inhibitors of the Na,K-ATPase, (3) we expected the two cardenolides to differ significantly in their inhibitory potency against the Na,K-ATPase of *O. fasciatus* due to differences in structure, steric conformation and lipophilicity.

Material and methods

Identification of sequences

Two of the three previously published gene copies of the Na,K-ATPase α 1 gene (Zhen et al., 2012) (α 1B Acc.No. JQ771519, α 1C Acc.No. JQ771518) were incomplete at the 3' end. Both missing ends could be recovered by RACE PCR (SMARTer RACE kit, Takara Bio, Mountain View, CA, USA).

Previously published transcriptomic data of *O. fasciatus* (Zhen et al., 2012) were used to identify the full-length coding sequences of the Na,K-ATPase β -subunits. The raw data were trimmed and assembled with Trinity (Grabherr et al., 2011) as described in Meinzer et al. (2019). *O. fasciatus* β -subunit sequences were recovered using published orthologous sequences from *Locusta migratoria* (Orthoptera; Acc.No. KF813098-KF813100) and *Blatta germanica* (Blattodea; Acc.No. HE819405) in tblastn searches against the transcriptome. *De novo* assembly of the raw reads and quality control was performed with the program CLC-Genomics Workbench (Qiagen, Hilden, Germany). The resulting *contigs* were screened for open reading frames (ORFs) with a length of roughly 900 bp (the consensus length of the highly conserved Na,K-ATPase β -subunit) using the program VectorNTI (Thermo Fisher, Darmstadt, Germany). Homology of the *contigs* were assigned by a further round of tblastx searches against published sequences in genbank.

Construction of recombinant baculoviruses

The coding sequences of the Na,K-ATPase α - and β -subunits genes (α 1A, α 1B, α 1C, β 1, β 2 and β 3) were amplified from cDNA transcribed with Superscript III (Thermo Fisher) from RNA extracted from nervous tissue, gut, testes or muscles with the RNAmagic-kit (Bio-Budget Technologies GmbH, Krefeld, Germany). For downstream cloning all subunits were

amplified with the Pfu-Phusion Polymerase (Thermo Scientific) and primers including the full coding sequence and incorporating suitable restriction sites at the 5' end.

PCR products were restricted with the chosen enzymes (Thermo Fisher), gel extracted (QIAquick gel extraction kit, Qiagen) and ligated into pFastBac-Dual vectors (Thermo Fisher). The final plasmids had an α -subunit under the control of the polyhedrin promoter (PH) and a β -subunit under the p10 promoter. Recombinant baculoviruses were generated as previously described (Dalla et al., 2017) and transfected into *Sf9* cells to obtain a first (P1) and second (P2) generation of recombinant virus stock.

Production of recombinant proteins

Production of different recombinant Na,K-ATPase α - β -subunit constructs started from infection of *Sf9* cells with P2 virus stock and followed our previously published protocol (Dalla et al., 2017). The cells were harvested by centrifugation, the pellet resuspended and sonicated and cell debris removed by centrifugation. Cell membranes were recovered by ultracentrifugation of the supernatant and redissolved in pure water (ROTIPURAN® p.a., ACS water; Roth). The concentrations of the resuspended protein pellets were determined by Bradford assay (Sigma-Aldrich, Taufkirchen, Germany) using bovine serum albumin (BSA) as a standard.

SDS-PAGE/ Western blotting

Thirty μ g of the heterologously expressed proteins were deglycosylated with endoglycosidase H following the manufacturer's protocol (Promega GmbH, Wiesloch, Germany) but instead of boiling proteins for 5 minutes at 95°C we performed the

denaturation step at 50°C for 30 minutes to maintain the integrity of the protein. SDS-polyacrylamide gel electrophoresis and western blotting was performed as previously reported (Dalla et al., 2017) with few modifications. The membrane proteins were blotted on a nitrocellulose membrane for 90 minutes at 100 V with an additional ice block at 4°C. To mask unspecific binding sites the membrane was blocked with BlueBlock PF (Serva, Heidelberg, Germany). The split membranes were incubated with the primary monoclonal antibodies $\alpha 5$, α -tubulin (DSHB Hybridoma Bank, University of Iowa; deposited by D.M. Fambrough (Lebovitz et al., 1989), and customized specific polyclonal antibodies for the subunits $\beta 1$, $\beta 2$ and $\beta 3$ (Davids Biotechnology, Regensburg, Germany). For detection of the primary antibodies the membrane was incubated for one hour with goat anti-mouse ($\alpha 5$, α -tubulin), anti-rabbit ($\beta 2$) and anti-chicken ($\beta 1$, $\beta 3$) secondary antibodies conjugated with horseradish peroxidase (Dianova, Hamburg, Germany) and developed with 4-chloro-1-naphtol as previously described (Dalla et al., 2017).

Extraction and preparation of cardenolides

Calotropin was extracted according to (Petschenka et al., 2018) from approximately 8 g of dried larvae of the monarch butterfly *Danaus plexippus* reared on *Asclepias curassavica*. The cardenolides were extracted with methanol, lipids extracted with hexane and the cardenolides retrieved by chloroform extraction. The concentrated methanolic extract was subjected to preparative HPLC (Agilent 1100) equipped with a photodiode array detector and a semi-preparative RP-Nucleodur C18 Htec column (5 μ m, 250 x 10 mm, Macherey-Nagel, Düren, Germany) using a water-acetonitrile gradient. Calotropin was identified by its retention time and checked for molecular weight and LS-MS spectrum by

LC-ESI-TOF spectrometry by Dr. Maria Riedner (Department of Chemistry, Universität Hamburg) and the structure confirmed by NMR spectroscopy. The resulting calotropin preparation was 80% pure.

Calotropin and the commercially available ouabain (Sigma-Aldrich) were first dissolved in DMSO and prepared for Na,K-ATPase activity assays as dilutions in sterile H₂O reaching final concentrations from 10⁻⁸ to 10⁻³ M with 2% DMSO. Ouabain could in addition be prepared as a 5x10⁻³ M solution.

Na,K-ATPase activity and inhibition assay

Activities of the nine different Na,K-ATPase α/β -subunit combinations of *O. fasciatus* were determined by photometric measurements of inorganic phosphate released from enzymatic ATP hydrolysis while subtracting background ATPase activity following previously described methods (Dalla et al., 2017; Petschenka et al., 2013). Based on previous results (Dalla et al., 2017) the buffer regime used was 50 mM NaCl, 4 mM MgCl₂, 50 mM imidazol, 20 mM KCl, pH 7.4. The inhibition of the different Na,K-ATPases was tested under increasing ouabain and calotropin concentrations and expressed as percentage activity of a non-inhibited control. For each α/β -subunit construct duplicates of at least three biological replicates of recombinant proteins isolated from independently transfected Sf9 cells were tested.

Analysis of data/ Statistics

After checking for normal distribution (Shapiro Wilk's test) and variance homogeneity (Levene's test on squared deviations), differences in total Na,K-ATPase activities were tested and compared between each α/β -subunit construct by one-way ANOVA followed by Tukey's HSD posthoc test. The data of each Na,K-ATPase α/β -subunit construct was subjected to nonlinear curve fitting with dose response function and Levenberg Marquardt iteration algorithm in Origin-Pro 9.1G (OriginLab Corporation, Northampton, USA) using a four parameter logistic function, top asymptote was set to 100, bottom asymptote set to 0 and a variable Hill slope. For each construct IC_{50} values were calculated and differences were analyzed separately for constructs affected by ouabain and calotropin by one-way ANOVA followed by Tukey's HSD posthoc tests. A pairwise, F-test based comparison of similarity between the fitted curves was performed using the command "compare datasets". All calculations were performed with Origin-Pro 9.1G.

Results

Bioinformatic analysis

Four potential β -subunits were identified from the *O. fasciatus* transcriptome and according to their similarity to published sequences named $\beta 1$, $\beta 2$, $\beta 3$ and βx (Genbank AccNo xxx-xxx). While $\beta 1$ -3 conform to the standard length of β -subunits of around 900 bp, βx is much shorter and missing most of the conserved N-terminal and half of the transmembrane region (Fig. S1A). This β -subunit was therefore not further considered

here. The 5' end of $\alpha 1B$ and $\alpha 1C$ could successfully be recovered by RACE protocols (Fig. S1B). The only minimally expressed $\alpha 1D$ (Yang et al., 2019) was not further investigated here.

Expression of α/β -subunit constructs in Sf9 cells

All nine α/β -subunit constructs ($\alpha A\beta 1$, $\alpha A\beta 2$, $\alpha A\beta 3$, $\alpha B\beta 1$, $\alpha B\beta 2$, $\alpha A\beta 3$, $\alpha C\beta 1$, $\alpha C\beta 2$, and $\alpha C\beta 3$) were successfully expressed in Sf9 cells and harvested. The western blot shows that identical concentrations were loaded onto the gel (α -tubulin as positive control), all α -subunits were expressed at similar levels and the deglycosylated β -subunits can be easily discriminated by their specific patterns (Fig. 2).

Na,K-ATPase activity of different α/β -subunit constructs

The activities of different α/β -subunit combinations of the Na,K-ATPase were tested *in vitro* to determine whether the amino acid substitutions in the cardenolide binding site or the associated β -subunit influence the catalytic activity of the enzyme. All enzymes showed catalytic activity, but the activity of the α/β -subunit constructs differed significantly from one another (Fig. 3). The α -subunits A and B with their associated β -subunits (1-3) showed significantly lower activity than αC combined with $\beta 1$, 2 or 3. The activities of αA and αB did not differ significantly from each other regardless of the β -subunit, except for $\alpha A\beta 2$. This combination was significantly less active than αB associated with the three β -subunits (Fig. 3). By far the highest activity was measured for αC in

association with $\beta 3$ that had an average turnover of $36.5 \text{ nmol Pi mg}^{-1} \text{ Protein} \cdot \text{minute}$ and differed significantly from all other constructs.

Influence of α/β -subunit association on ouabain sensitivity

In vitro tests with the cardenolide ouabain revealed large differences in sensitivity of the Na,K-ATPases depending on the number of amino acid substitutions in the cardenolide binding site and was modulated by the identity of the β -subunit (Fig. 4). Yet, when comparing the IC_{50} values only the one of αC associated with $\beta 1$ was significantly lower than those for the other two αC constructs (ANOVA, $F_{2,8}=8.035$, $p=0.02$, Tukey's HSD, $p=0.035$ and $p=0.027$, for $\alpha C \beta 1$ vs $\beta 2$ and $\beta 1$ vs $\beta 3$, respectively; Fig. 4c, Table 1) whereas the IC_{50} values of the three αA or αB constructs did not differ significantly.

Although analyzing differences in IC_{50} is a standard approach, the IC_{50} values only reflect one point of the dose response curve. We therefore aimed to get a better insight into the overall responses of the Na,K-ATPases to increasing ouabain concentrations by analyzing the differences between the full dose response curves of constructs with the same α -subunit as well as constructs with the same β -subunit (Table 2).

We found that α -subunits associated with different β -subunits behaved differently when subjected to ouabain. The construct $\alpha A \beta 3$ was significantly more sensitive to the cardenolide than the other two αA complexes (Table 2, Fig. 4a), and $\alpha B \beta 1$ was inhibited more strongly by ouabain than $\alpha B \beta 2$ (Table 2, Fig. 4b). Nevertheless, the reaction of constructs with identical α -subunits was more uniform than the one of constructs with identical β - but different α -subunits. Most of the latter constructs differed significantly in

their reactions, reflecting their varying insensitivity levels against ouabain (Fig. 4 a-c, Table 2).

Influence of α/β -subunit association on calotropin sensitivity

Testing the inhibitory effect of the host plant cardenolide calotropin on the recombinant Na,K-ATPases resulted in total inhibition of the most calotropin-sensitive $\alpha C/\beta$ -subunit constructs at the highest calotropin concentration. Furthermore, the three αB constructs turned out to be most resistant against calotropin. As for the inhibition by ouabain only the IC_{50} values of αC associated with the three different β -subunits differed significantly from each other, αC associated with $\beta 3$ was significantly more insensitive than αC associated with $\beta 1$ and $\beta 2$ (ANOVA, $F_{2,8}=18.554$, $p=0.003$, Tukey's HSD, $p=0.002$ and $p=0.027$, for $\alpha C\beta 3$ versus $\alpha C\beta 1$ and $\alpha C\beta 3$ versus $\alpha C\beta 2$, respectively; Fig. 4c).

When comparing the full dose response curves of the constructs (Table 2) neither the three αA constructs differed significantly from one another nor the αB or αC constructs, indicating a similar response to calotropin exposition independent of the associated β -subunit (Fig. 4 a-c). In contrast, the inhibition by calotropin of the three α -subunits combined with the same β -subunits was significantly different (from high to low inhibition: $\alpha C > \alpha A > \alpha B$; Fig. 4a-c). The curves of $\alpha A\beta 1$ and $\alpha B\beta 1$ did not differ significantly from one another, since $\alpha A\beta 1$ tends to be the most resistant combination of the three αA constructs and $\alpha B\beta 1$ the most sensitive of the three αB constructs (Fig. 4a & b).

Comparison of both cardenolides

Calotropin had a higher inhibitory potency and inhibited especially the three α C-constructs to a much higher extent than ouabain (ANOVA, $F_{5,17}=57.25882$, $p<0.001$, Tukey's HSD, $p<0.001$ for α C β 1, α C β 2 and α C β 3, respectively; Fig. 4c, Table 1). Calotropin inhibited α C β 1 18-fold, α C β 2 20-fold and α C β 3 14-fold more strongly than ouabain did. Similarly, α B associated with β 3 was significantly more inhibited by calotropin than by ouabain, resulting in an approximately 24-fold higher IC_{50} (ANOVA, $F_{5,17}=7.54487$, $p=0.00205$, Tukey's HSD, $p=0.00357$; Fig. 4b, Table 1) while the IC_{50} values of the two cardenolides did not differ significantly for α B β 1 and α B β 2 (Tukey's HSD, $p=0.70595$ and $p=0.51146$, respectively; Fig. 4b, Table 1).

Additionally, we compared the dose response curves of same constructs inhibited by calotropin and ouabain. Besides similar results for the α C constructs, we found that the construct α A β 1 was significantly more insensitive against calotropin than against ouabain, reflected by a flat descending curve at the top (Fig. 4c & 4a, Table 3).

Table 1: Mean IC_{50} values and Hill slopes of different Na,K-ATPase α - β -constructs (3 biological replicates with standard deviation ($\pm$ s.d.)) obtained from dose response curves upon inhibition with ouabain and calotropin (Origin-Pro 9.1G).

α/β - combination	IC_{50} values [M] Ouabain ($\pm$ s.d.)	Hill slope ($\pm$ s.d.)	IC_{50} values [M] Calotropin ($\pm$ s.d.)	Hill slope ($\pm$ s.d.)

$\alpha A\beta 1$	4.148E-04 ($\pm 6.541E-04$)	-0.221 (± 0.047)	1.211E-04 ($\pm 4.443E-05$)	-0.271 (± 0.03)
$\alpha A\beta 2$	3.471E-04 ($\pm 3.598E-04$)	-0.234 (± 0.013)	1.449E-05 ($\pm 5.994E-06$)	-0.269 (± 0.076)
$\alpha A\beta 3$	2.607E-05 ($\pm 2.767E-05$)	-0.260 (± 0.044)	6.959E-05 ($\pm 7.54E-05$)	-0.27 (± 0.036)
$\alpha B\beta 1$	2.089E-03 ($\pm 2.515E-03$)	-0.213 (± 0.039)	1.108E-04 ($\pm 3.423E-05$)	-0.293 (± 0.044)
$\alpha B\beta 2$	2.777E-03 ($\pm 9.676E-04$)	-0.462 (± 0.055)	3.281E-04 ($\pm 2.119E-04$)	-0.398 (± 0.066)
$\alpha B\beta 3$	7.09E-03 ($\pm 3.119E-03$)	-0.261 (± 0.045)	2.944E-04 ($\pm 8.195E-05$)	-0.254 (± 0.071)
$\alpha C\beta 1$	4.274E-04 ($\pm 8.104E-05$)	-0.869 (± 0.071)	2.402E-05 ($\pm 4.636E-06$)	-0.88 (± 0.029)
$\alpha C\beta 2$	7.369E-04 ($\pm 1.001E-04$)	-0.869 (± 0.093)	3.699E-05 ($\pm 5.517E-06$)	-1.289 (± 0.198)
$\alpha C\beta 3$	7.579E-04 ($\pm 1.476E-04$)	-0.988 (± 0.35)	5.594E-05 ($\pm 8.55E-06$)	-0.912 (± 0.098)

319 Table 2: Comparison between the dose response curves of different α/β constructs inhibited by ouabain and
320 calotropin analyzed with Origin-Pro 9.1G. Significant differences are highlighted in bold.

Data comparison	F-statistic	F-statistic
α/β -combinations	Ouabain	Calotropin
$\alpha A\beta 1 - \alpha A\beta 2$	$F_{2,8} = 0.074, p = 0.929$	$F_{2,8} = 1.319, p = 0.320$
$\alpha A\beta 1 - \alpha A\beta 3$	$F_{2,8} = 5.218, p = 0.035$	$F_{2,8} = 0.728, p = 0.512$
$\alpha A\beta 2 - \alpha A\beta 3$	$F_{2,8} = 6.092, p = 0.025$	$F_{2,8} = 0.079, p = 0.925$
$\alpha B\beta 1 - \alpha B\beta 2$	$F_{2,8} = 10.981, p = 0.005$	$F_{2,8} = 2.607, p = 0.134$
$\alpha B\beta 1 - \alpha B\beta 3$	$F_{2,8} = 3.781, p = 0.070$	$F_{2,8} = 0.724, p = 0.514$
$\alpha B\beta 2 - \alpha B\beta 3$	$F_{2,8} = 0.455, p = 0.650$	$F_{2,8} = 0.655, p = 0.545$
$\alpha C\beta 1 - \alpha C\beta 2$	$F_{2,8} = 0.745, p = 0.505$	$F_{2,8} = 1.887, p = 0.213$
$\alpha C\beta 1 - \alpha C\beta 3$	$F_{2,8} = 1.535, p = 0.273$	$F_{2,8} = 3.402, p = 0.085$
$\alpha C\beta 2 - \alpha C\beta 3$	$F_{2,8} = 1.080, p = 0.384$	$F_{2,8} = 2.217, p = 0.171$
$\alpha A\beta 1 - \alpha B\beta 1$	$F_{2,8} = 1.039, p = 0.397$	$F_{2,8} = 0.554, p = 0.595$
$\alpha A\beta 1 - \alpha C\beta 1$	$F_{2,8} = 22.729, p < 0.001$	$F_{2,8} = 8.724, p = 0.010$
$\alpha B\beta 1 - \alpha C\beta 1$	$F_{2,8} = 11.975, p = 0.004$	$F_{2,8} = 8.795, p = 0.010$
$\alpha A\beta 2 - \alpha B\beta 2$	$F_{2,8} = 57.311, p < 0.001$	$F_{2,8} = 9.763, p = 0.007$
$\alpha A\beta 2 - \alpha C\beta 2$	$F_{2,8} = 4.835, p = 0.042$	$F_{2,8} = 6.422, p = 0.022$
$\alpha B\beta 2 - \alpha C\beta 2$	$F_{2,8} = 2.353, p = 0.157$	$F_{2,8} = 13.589, p = 0.003$
$\alpha A\beta 3 - \alpha B\beta 3$	$F_{2,8} = 32.590, p < 0.001$	$F_{2,8} = 37.553, p < 0.001$
$\alpha A\beta 3 - \alpha C\beta 3$	$F_{2,8} = 33.295, p < 0.001$	$F_{2,8} = 19.271, p < 0.001$

$\alpha\text{B}\beta 3 - \alpha\text{C}\beta 3$	$F_{2,8} = 1.614, p = 0.258$	$F_{2,8} = \mathbf{8.239}, p = \mathbf{0.011}$

321

322 Table 3: Comparison between dose response curves of same α/β constructs inhibited by ouabain and

323 calotropin analyzed with Origin-Pro 9.1G. Significant results are highlighted in bold.

Data comparison α/β -combinations	F-statistic calotropin vs. ouabain
$\alpha\text{A}\beta 1$	$F_{2,8} = \mathbf{11.752}, p = \mathbf{0.004}$
$\alpha\text{A}\beta 2$	$F_{2,8} = 2.421, p = 0.151$
$\alpha\text{A}\beta 3$	$F_{2,8} = 0.589, p = 0.577$
$\alpha\text{B}\beta 1$	$F_{2,8} = 0.343, p = 0.720$
$\alpha\text{B}\beta 2$	$F_{2,8} = 4.257, p = 0.055$
$\alpha\text{B}\beta 3$	$F_{2,8} = 3.054, p = 0.103$
$\alpha\text{C}\beta 1$	$F_{2,8} = \mathbf{35.226}, p < \mathbf{0.001}$
$\alpha\text{C}\beta 2$	$F_{2,8} = \mathbf{100.356}, p < \mathbf{0.001}$
$\alpha\text{C}\beta 3$	$F_{2,8} = \mathbf{74.665}, p < \mathbf{0.001}$

324

325 Discussion

326 Here we have identified the full length coding sequences of three Na,K-ATPase α - and
 327 three full length β -subunits of *O. fasciatus* and functionally characterized all nine possible
 328 recombinant α/β enzyme complexes. These nine functional enzymes strongly differed in
 329 their activity levels. We found that enzyme complexes with αC had a significantly higher

activity compared to the other constructs. This perfectly fits with the fact that αC is mainly expressed in the nervous tissue of *O. fasciatus* (Lohr et al., 2017; Zhen et al., 2012) where the Na,K-ATPase plays a crucial role in maintaining electric potentials that guarantee the excitability of neurons. Consequently, a high ionic pumping activity is indispensable. Two of our nine α/β constructs clearly stand out amongst the other enzyme complexes: $\alpha A\beta 2$ and $\alpha C\beta 3$. The activity of $\alpha A\beta 2$ is reduced in comparison to the other αA and αB constructs that all show similar activity levels. Therefore, we suppose that $\alpha A\beta 2$ is not a functional enzyme *in vivo*. In contrast, the activity of αC combined with $\beta 3$ is significantly higher and would therefore well fit with a natural occurrence of this complex in the nervous tissue. Data of an ongoing proteomic study support this assumption and show that the subunits αC and $\beta 3$ are most abundant in the nervous tissue of *O. fasciatus* (M. Winter et al., unpublished data).

Regardless of the strikingly high overall resistance to inhibition by cardenolides that Na,K-ATPase preparations of *O. fasciatus* display (Bramer et al., 2015; Dalla and Dobler, 2016; Moore and Scudder, 1986) we found that the individual α/β -combinations strongly differed in their resistance against the two cardenolides ouabain and calotropin. Overall calotropin proved to be the much more potent inhibitor. This is despite the fact that *O. fasciatus* is naturally exposed to calotropin in its host plants (Malcolm, 1991) and thus could be specifically adapted to this toxin. Yet, our data demonstrate that in particular the most active αC -subunit was roughly 20-times more strongly inhibited by calotropin than by ouabain. A differential inhibition can also be observed for the three αB constructs while the difference in inhibition between ouabain and calotropin is least pronounced for the

α A constructs. Overall, the α A- and α B-subunits are more insensitive to both cardenolides than α C, which fits well with their abundant expression in the Malpighian tubules where the enzymes are directly exposed to toxins in the hemolymph (Lohr et al., 2017). In contrast, the least sensitive but most active α C is the dominant Na,K-ATPase α -subunit in the nervous tissue where it is protected from intruding cardenolides by the perineurium (blood-brain-barrier) which controls xenobiotica access to the nervous system by a tight network of septate junctions and active efflux carriers (Dermauw and Van Leeuwen, 2014).

The difference in inhibitory potencies of the two cardenolides results from differences in their structure, steric conformation and - *in vivo* - by their ability to permeate membranes, as reflected by their lipophilicity. Ouabain is produced in plants of the genus *Acokanthera* and in seeds of *Strophanthus gratus* (both Apocynaceae), whereas calotropin is synthesized in plants of the tribe Asclepiadeae (Apocynaceae). Cardiotonic activity is determined by the steroidal aglycone, specifically by the conformation of the steroid rings. The A/B and C/D rings of ouabain, and many other cardenolides, are *cis*-fused and the B/C ring is *trans*-fused (*cis-trans-cis*) (Katz et al., 2010; Malcolm, 1991). In contrast calotropin and other cardenolides produced in the genus *Asclepias*, have a *trans-trans-cis* conformation (Malcolm, 1991) that is predicted to lower the toxicity (Hoch, 1961) and the binding affinity of the cardenolide (Katz et al., 2010). However, a doubly-linked sugar moiety, often found in cardenolides produced in plants of the tribe Asclepiadeae, enhances the binding to the Na,K-ATPase as well as the toxicity (Piacente et al., 2009; Venkateswara Rao and Vaidyanathan, 1991). Despite their differences in structure and

steric conformation, molecular docking analyses suggest that both cardenolides fit well into the cardenolide binding site due to formation of strong H-bonds between the interaction partners (Zhang et al., 2014). Similar to our results a study testing various cardiac glycosides against the Na,K-ATPase of the monarch butterfly, *Danaus plexippus*, (Petschenka et al., 2018) found calotropin and its isomer calactin to be the strongest tested inhibitors, with calotropin inhibiting 60-fold more strongly than ouabain.

A comparison of the present results with our previous studies (Dalla and Dobler, 2016; Dalla et al., 2017) on *O. fasciatus* Na,K-ATPase mimics reveals good concordance regarding our conclusions about enzyme activities. We previously introduced one, two, three or four of the potentially resistance-conferring substitutions into the α -subunit of *D. melanogaster* to mimic the known α 1-gene copies of *O. fasciatus* (in this order: α D, α C, α B, α A-mimic; c.f. Yang et al., 2019; Zhen et al., 2012) and combined them with two *D. melanogaster* β -subunits, Nrv2.2 and Nrv3 (Dalla and Dobler, 2016; Dalla et al., 2017). The overall behavior of these mimics strongly resembled the present data for the original *O. fasciatus* Na,K-ATPase complexes. Most strikingly, the activity levels of the α A and α B mimics were significantly lower than of the *Drosophila* wildtype enzyme, the α C or the α D (N122H) mimics. The two *Drosophila* β -subunits also modulated the activity: the association with Nrv3 strongly reduced the activity of all mimics and the *Drosophila* wildtype enzyme when compared to those bearing Nrv2.2. In the present study, such a large influence of the β -subunit on ion pumping activity was observed only in the α C-subunit while the effect was less pronounced for the α A and α B-subunits.

The β -subunits also strongly modulated the resistance of the enzymes against ouabain, but their effect was specific to the α -subunits they were combined with. Previous work has shown that a combination with Nrv3 instead of Nrv2.2 lead to lowered resistance in the α A- and α B-mimic and increased resistance in the α C-mimic (Dalla et al., 2017). In the present study, resistance changed in the same direction with β 1, β 2, or β 3 for α A (decreasing) and α C (increasing IC_{50}) but also lead to increased IC_{50} -values for α B.

The most striking differences, however, appear when comparing the overall ouabain-sensitivity of the original enzyme combinations with the previously constructed mimics (Dalla et al., 2017). While the mimics of the *O. fasciatus* α A-copy (featuring the substitutions Q111T,N122H,F786N,T797A) were by far the most insensitive constructs followed by the α B-mimics (with Q111T,N122H,F786N) and the more sensitive α C-mimics (N122H,F786S), the original α A-constructs tested here are at least five times less sensitive than α B-constructs. On the other hand, the original α C-constructs are 10-times more resistant than predicted from the experiments with the α C-mimics.

These differences in sensitivity between mimics and original enzymes strongly point to the importance of additional substitutions in the *O. fasciatus* Na,K-ATPase α 1-paralogs. When constructing the mimics, we focused on the four most conspicuous amino acid exchanges in the binding pocket of the Na,K-ATPase that were previously shown functionally to be essential for cardiac glycoside binding (Croyle et al., 1997; Holzinger et al., 1992; Qiu et al., 2005) and confirmed to interact with cardiac glycosides by crystal structure analysis (Laursen et al., 2015). However, there are additional amino acid substitutions in the cardiac glycoside binding pocket of the Na,K-ATPase α 1-genes of *O. fasciatus* that may

further increase their resistance (Dobler et al., 2012; Zhen et al., 2012). These and further additional amino acid exchanges that at first appear inconsequential may be of importance in modulating the effects of the resistance conferring substitutions or ameliorating their pleiotropic effects (Karageorgi et al., 2019; Taverner et al., 2019; Mohammadi et al., submitted).

Author contribution - S. Da. and S. Do. conceptualization; S. Da., M. W., V. W., R. T., M. H. cloning and protein expression; S. Da. and M. W. enzyme assays, data analysis and visualization; M. W. and S. Do. writing-original draft; S. Da. writing-review and editing.

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Figure 1: Structure of the cardenolides ouabain (a) and calotropin (b). Both are characterized by a monosaccharide attached to a steroid skeleton at C3 (4 steroid rings: A-D) and a five-membered unsaturated lactone ring at C-17. a) In ouabain the monosaccharide is attached by a single glycosidic link to the steroid rings that are *cis-trans-cis* fused. b) Calotropin is characterized by a doubly-linked monosaccharide as well as a *trans-trans-cis* conformation of the steroid rings (structures generated by Daniel Tal, The Weizmann Institute of Science, 2020).

Figure 2: Western blot of nine α/β constructs of *O. fasciatus* expressed heterologously in cell culture. 30 μ g of protein were separated by SDS-PAGE and blotted on a nitrocellulose membrane. The upper membrane slice shows at a size of 110 kDa αA , αB and αC in alternating order. At 55 kDa α -tubulin is shown as a positive control. Both proteins were stained with the primary antibody $\alpha 5$ (DSHB hybridoma bank) and the secondary HRP-conjugated antibody goat-anti mouse (Dianova). The associated β -subunits 1, 2 and 3 were deglycosylated in advance. They were stained with β -subunit specific customized primary antibodies (produced by Davids Biotechnology) and HRP-conjugated secondary antibodies goat-anti rabbit (for $\beta 2$, Dianova) and goat-anti chicken (for $\beta 1$ and 3, Dianova). The different β -subunits show bands at weights ranging between 35 and 40 kDa. The bands were visualized with 4-CN. Due to the grey scale some bands of the multicolor protein ladder were marked by lines.

Figure 3: Na,K-ATPase activity [nmol Pi/mg protein*min] of nine α/β -constructs of *O. fasciatus*. Each bar depicts the mean and standard deviation of three biological replicates. Different letters above the bars indicate significant differences between constructs (ANOVA, $F_{8,28}=63.932$, $p<0.001$). Black bars: αA with three β -subunits and overall activities ranging from 7 to 12 nmol Pi/mg protein*min. Bars with a grey dotted outline: data for the αA mimic (THNA) combined with Nrv2.2 and Nrv3 of a previous study (24). Dark gray bars: $\alpha B/\beta$ constructs ranging from 13 to 14.3 nmol Pi/mg protein*min. Light gray bars: $\alpha C/\beta$ constructs with activity levels ranging from 22 to 36.5 nmol Pi/mg protein*min. The activity levels of the three $\alpha C/\beta$ constructs are significantly higher than those of the other constructs, with the highest activity reached by αC combined with $\beta 3$.

Figure 4: *In vitro* inhibition by calotropin and ouabain of nine α/β constructs of *O. fasciatus* expressed heterologously in cell culture. Dose response relationships between increasing cardenolide concentrations and residual enzyme activities were expressed in % of the non-inhibited control (calculated with Origin Pro 9.1G). Each data point represent the mean of three biological replicates and standard deviation. a) effect of calotropin (blue curves and round symbols) and ouabain (black curves and square symbols) on αA , b) on αB , and c) on αC combined each with $\beta 1$, 2 and 3. Please note the different scales.