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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Abstract

Background

Cardiac glycosides are known to fatally inhibit the Na,K-ATPase throughout the animal kingdom. Several animals, however, evolved target-site insensitivity by substitutions in the otherwise highly conserved cardiac glycoside binding pocket located on the Na,K-ATPase α-subunit. The minimal functional enzyme consist of an α- and a β-subunit, the latter 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 Na,K-ATPase gene duplications followed by differential resistance conferring substitutions and subfunctionalization of the enzymes.

Results

To investigate their characteristics we expressed nine combinations of *O. fasciatus* Na,K-ATPase α/β-sunbunits (three each) in *Sf*9 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 (αCβ3) or altered resistances. The host plant toxin calotropin proved to be a much more potent inhibitor than ouabain for the

45 phylogenetically oldest αC based enzymes. This effect was compensated for in the αB and
46 αA based enzymes with αAβ1 having higher resistance against calotropin than against
47 ouabain.

48 Conclusion

49 The originally higher inhibitory potency of the host compound calotropin supports a
50 coevolutionary escalation of plant defenses and herbivore tolerance mechanisms. For the
51 bugs the possession of multiple paralogs improved adaptation to plant toxins in a stepwise
52 manner and mitigates pleiotropic effects by a compromise between ion pumping activity
53 and resistance.

54

55 **Background**

56 The transmembrane Na,K-ATPase, is ubiquitously expressed in animal cells and is essential
57 for maintaining membrane potentials, controlling cell volume and the overall functionality
58 of cells (1). The functional enzyme consists of two subunits, the catalytic α-subunit and an
59 associated β-subunit serving as chaperone for correct folding and membrane integration
60 (2). In vertebrates several paralogs are present for both genes and the enzyme's
61 characteristics differ depending on the specific combination of α and β-subunits (3,4). An
62 additional modulatory and tissue specific γ-subunit is present in vertebrate tissues (5), but
63 has not been detected in invertebrates. Much less is known about the importance of
64 different α/β-subunit combinations in invertebrates (6). Only in *Drosophila* the tissue
65 distribution and functional roles of some of these combinations have been explored to

66 some extent (7–9). We here explore the functional role of different Na,K-ATPase
67 complexes in the large milkweed bug, *Oncopeltus fasciatus*. In this species, the Na,K-
68 ATPase α1 subunit has undergone three rounds of duplications (10,11) and displays high
69 insensitivity against cardiac glycosides (12,13), which are universal inhibitors of the Na,K-
70 ATPase (1).

71 Inhibition of the Na,K-ATPase by cardiac glycosides, such as the cardenolides ouabain and
72 calotropin (Fig. 1), depends on a specific binding site located on the extracellular side of
73 the catalytic α-subunit. Upon binding of cardiac glycosides the enzyme is arrested in the
74 phosphorylated state and ion pumping is disrupted (1). Inhibition of the Na,K-ATPase is
75 harmful and can be lethal to almost all animals. There are, however, a few notable
76 exceptions to this: various mammals, frogs, and reptiles prey unharmed on cardiac
77 glycoside-containing toads and a number of phytophagous insects feed - often exclusively
78 - on cardiac glycoside-containing host plants (reviewed in 14,15). Most of these species
79 avoid the toxic effect of cardiac glycosides and exhibit target site insensitivity, by
80 drastically lowering binding affinity of cardiac glycosides to their Na,K-ATPase α-subunit
81 (16,17). Strikingly, identical amino acid substitutions at homologous positions
82 convergently evolved in various, distantly related insect groups and even resemble the
83 substitutions observed in cardiac glycoside-resistant vertebrates (11,17–19).

84 While insect species usually express a single gene copy of the Na,K-ATPase α-subunit
85 (ATPα1) throughout their body, this gene underwent one to three rounds of gene
86 duplications in several cardiac glycoside-exposed species (10,11,16,20). These duplicated

87 ATPα1 copies differ in the number and identity of presumably resistance-causing amino
88 acid substitutions and show tissue specific expression patterns (10,11,20,21).

89 The large milkweed bug *Oncopeltus fasciatus* (Hemiptera, Lygaeidae) possesses four
90 ATPα1 paralogs (A, B, C, D) that differ in the number and identity of amino acid
91 substitutions (10,11). We previously introduced substitutions observed in these paralogs
92 at four usually highly conserved amino acid sites (Q111, N122, F786, and T797) known to
93 be important for high affinity to cardiac glycosides (22–25) into the sensitive ATPα1 gene
94 of *Drosophila melanogaster* (26,27). The N122H (as observed in ATPα1D of *O. fasciatus*),
95 N122H+T797S (as observed in ATPα1C), Q111T+N122H+F786N (as in ATPα1B) or
96 Q111T+N122H+F786N+T797A (as in ATPα1A) substitutions yielded proteins with stepwise
97 increasing resistance to the cardenolide ouabain. At the same time, enzyme activity
98 decreased with increasing numbers of substitutions (26).

99 Additional untested substitutions are present in the *O. fasciatus* ATPα1 paralogs and these
100 might influence the characteristics of the enzymes, potentially further increasing
101 resistance but possibly also serving as compensatory mutations mitigating deleterious
102 intra-molecular epistatic effects of previously characterized mutations (28–30). In
103 addition, it has been shown that the β-subunit of the Na,K-ATPase not only acts as a
104 chaperone, guiding the heterodimeric enzyme to the cell membrane, but also influences
105 the behavior of the Na,K-ATPase including its sensitivity to ouabain (2,4,27).

106 Although ouabain is a commonly used cardiac glycoside in studies of the Na,K-ATPase, it
107 does not occur in host plants of *O. fasciatus*. The *Asclepias* species (Apocynaceae) that the
108 bugs feed on typically contain different types of cardenolides characterized by a *trans*-

109 *trans-cis* conformation of the steroidal skeleton and a single sugar moiety attached by a
110 cyclic bridge as in the abundant calotropin (15) (Fig. 1). The most biologically relevant test
111 of the bugs' resistance against cardiac glycosides should thus involve a plant-derived
112 compound like calotropin. To address these issues, we identified and heterologously
113 expressed nine possible *O. fasciatus* Na,K-ATPase α/β combinations and characterized
114 their activities and sensitivities to ouabain and calotropin.

115 Since activity and resistance of the Na,K-ATPase is determined by the number and identity
116 of amino acid substitutions in the cardenolide binding site located in the α-subunit, (1) we
117 hypothesized that α-subunit paralogs – associated with different β-subunits – differ
118 significantly in their activities and insensitivities to the two cardenolides we tested. (2) We
119 further hypothesized that the different β-subunits modulate the Na,K-ATPase α-subunit
120 paralog behavior influencing their response to the cardenolides. Additionally, although
121 ouabain and calotropin both seem to be strong inhibitors of the Na,K-ATPase, (3) we
122 expected the two cardenolides to differ significantly in their inhibitory potency against the
123 Na,K-ATPase of *O. fasciatus* due to differences in structure and steric conformation. We
124 hypothesized that coevolutionary interaction should lead to higher resistance against the
125 host plant compound calotropin.

126

127

128 **Results**

129 *Bioinformatic analysis*

130 Four potential β-subunits were identified from the *O. fasciatus* transcriptome and
131 according to their similarity to published sequences named β1, β2, β3, and βx (Genbank
132 AccNo xxx-xxx). While β1-3 conform to the standard length of β-subunits of around 900 bp
133 and are recovered in Blast searches as homologues of similarly numbered β-subunits of
134 other insects, βx is much shorter and missing most of the conserved N-terminal and half of
135 the transmembrane region and does not have obvious homologues in other insects (Fig.
136 S1A). This β-subunit was therefore not further considered here. The 5' end of α1B and α1C
137 could successfully be recovered by RACE protocols (Genbank AccNo xxx, xxx). The only
138 minimally expressed α1D (10) was not further investigated here.

139

140 *Expression of α/β-subunit constructs in* Sf*9 cells*

141 All nine α/β-subunit constructs (αAβ1, αAβ2, αAβ3, αBβ1, αBβ2, αAβ3, αCβ1, αCβ2, and
142 αCβ3) were successfully expressed in *Sf*9 cells and the corresponding proteins harvested.
143 The western blot shows that similar concentrations were loaded onto the gel (12G10 anti-
144 α-tubulin as positive control), all α-subunits were expressed at similar levels and the
145 deglycosylated β-subunits can be easily discriminated by their specific patterns (Fig. 2).

146 *Na,K-ATPase activity of different α/β-complexes*

147 The activities of different α/β-subunit combinations of the Na,K-ATPase were tested *in*
148 *vitro* to determine whether the amino acid substitutions in the cardenolide binding site or
149 the associated β-subunit influence the catalytic activity of the enzyme. All enzymes
150 showed catalytic activity, but the activity of the α/β-complexes differed significantly from

151 one another (Fig. 3). The α-subunits A and B with their associated β-subunits (1-3) showed
152 significantly lower activity than αC combined with β1, 2 or 3. The activities of αA and αB
153 did not differ significantly from each other regardless of the β-subunit, except for αAβ2.
154 This combination was significantly less active than αB associated with the three β-subunits
155 (Fig. 3). By far the highest activity was measured for αC in association with β3 that had an
156 average turnover of 32.7 nmol Pi per mg Protein per minute and differed significantly
157 from all other enzymes.

158 *Influence of α/β-subunit combination on ouabain sensitivity*

159 *In vitro* tests with the cardenolide ouabain revealed large differences in sensitivity of the
160 Na,K-ATPases depending on the number of amino acid substitutions in the cardenolide
161 binding site and was modulated by the identity of the β-subunit (Fig. 4). Yet, when
162 comparing the IC_{50} values only the one of αC associated with β1 was significantly lower
163 than those for the other two αC complexes (ANOVA, $F_{2,8}$=8.035, p=0.02, Tukey's HSD,
164 p=0.035 and p=0.027, for αC β1 vs β2 and β1 vs β3, respectively; Fig. 4c, Table 1) whereas
165 the IC_{50} values of the three αA or αB complexes did not differ significantly.

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

171 We found that α-subunits associated with different β-subunits behaved differently when
172 subjected to ouabain. The enzyme αAβ3 was significantly more sensitive to the
173 cardenolide than the other two αA complexes (Table 2, Fig. 4a), and αBβ1 was inhibited
174 more strongly by ouabain than αBβ2 (Table 2, Fig. 4b). Nevertheless, the reaction of
175 enzymes with identical α-subunits was more uniform than the one of Na,K-ATPases with
176 identical β but different α-subunits. Most of the latter enzymes differed significantly in
177 their reactions, reflecting their varying insensitivity levels against ouabain (Fig. 4 a-c, Table
178 2).

179

180 *Influence of α/β-subunit association on calotropin sensitivity*

181 Testing the inhibitory effect of the host plant cardenolide calotropin on the recombinant
182 Na,K-ATPases resulted in total inhibition of the most calotropin-sensitive αC/β-
183 combinations at the highest calotropin concentration. Furthermore, the three αB based
184 enzymes turned out to be most resistant against calotropin. As for the inhibition by
185 ouabain only the IC_{50} values of αC associated with the three different β-subunits differed
186 significantly from each other, αC associated with β3 was significantly more insensitive to
187 calotropin than αC associated with β1 and β2 (ANOVA, $F_{2,8}$=18.554, p=0.003, Tukey's HSD,
188 p=0.002 and p=0.027, for αCβ3 versus αCβ1 and αCβ3 versus αCβ2, respectively; Fig. 4c).

189 When comparing the full dose response curves of the constructs (Table 2) neither the
190 three αA combinations differed significantly from one another nor the αB or αC based
191 enzymes, indicating a similar response to calotropin exposition independent of the

192 associated β-subunit (Fig. 4 a-c). In contrast, the inhibition by calotropin of the three α-
193 subunits combined with the same β-subunits was significantly different (from high to low
194 inhibition: αC> αA> αB; Fig. 4a-c). The curves of αAβ1 and αBβ1 did not differ significantly
195 from one another, since αAβ1 tends to be the most resistant combination of the three αA
196 enzymes and αBβ1 the most sensitive of the three αB enzymes (Fig. 4a & b).

197

198 *Comparison of both cardenolides*

199 Calotropin inhibited especially the three αC enzymes to a much higher extent than
200 ouabain as judged by a comparison of IC_{50} values (ANOVA, $F_{5,17}$=57.25882, p<0.001,
201 Tukey's HSD, p<0.001 for all αC based enzymes; Fig. 4c, Table 1). αCβ1 was inhibited 18-
202 fold, αCβ2 20-fold, and αCβ3 14-fold more strongly by calotropin than by ouabain. When
203 only comparing IC_{50} values αB associated with β3 was also significantly more strongly
204 inhibited by calotropin than ouabain, resulting in an approximately 24-fold higher IC_{50}
205 (ANOVA, $F_{5,17}$=7.54487, p=0.00205, Tukey's HSD, p=0.00357; Fig. 4b, Table 1) while the
206 IC_{50} values of the two cardenolides did not differ significantly for αBβ1 and αBβ2 (Tukey's
207 HSD, p=0.70595 and p=0.51146, respectively; Fig. 4b, Table 1). Comparison of the two
208 cardenolides resulted in no significant differences between IC_{50} values of αA associated
209 with any of the three β-subunits (ANOVA, $F_{5,16}$=1.85583, p=0.18241; Fig. 4b, Table 1).

210 However, when comparing the full dose response curves of each enzyme inhibited by
211 either of the cardenolides, we found that αAβ1 was significantly more insensitive against
212 calotropin than against ouabain, reflected by a flat descending curve at the top (Fig. 4a,

213 Table 3). For the αC-based enzymes the stronger inhibition by calotropin remained
214 evident, while for enzymes with αB no difference in inhibitory potency between the two
215 cardenolides was found (Table 3).

216

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

α/β-combination	IC_{50} values [M] Ouabain (± s.d.)	Hill slope (± s.d.)	IC_{50} values [M] Calotropin (± s.d.)	Hill slope (± s.d.)
αAβ1	4.148E-04 (± 6.541E-04)	-0.221 (± 0.047)	1.211E-04 (± 4.443E-05)	-0.271 (± 0.03)
αAβ2	3.471E-04 (± 3.598E-04)	-0.234 (± 0.013)	1.449E-05 (± 5.994E-06)	-0.269 (± 0.076)
αAβ3	2.607E-05 (± 2.767E-05)	-0.260 (± 0.044)	6.959E-05 (± 7.54E-05)	-0.27 (± 0.036)
αBβ1	2.089E-03 (± 2.515E-03)	-0.213 (± 0.039)	1.108E-04 (± 3.423E-05)	-0.293 (± 0.044)
αBβ2	2.777E-03 (± 9.676E-04)	-0.462 (± 0.055)	3.281E-04 (± 2.119E-04)	-0.398 (± 0.066)

αBβ3	7.09E-03 (± 3.119E-03)	-0.261 (± 0.045)	2.944E-04 (± 8.195E-05)	-0.254 (± 0.071)
αCβ1	4.274E-04 (± 8.104E-05)	-0.869 (± 0.071)	2.402E-05 (± 4.636E-06)	-0.88 (± 0.029)
αCβ2	7.369E-04 (± 1.001E-04)	-0.869 (± 0.093)	3.699E-05 (± 5.517E-06)	-1.289 (± 0.198)
αCβ3	7.579E-04 (± 1.476E-04)	-0.988 (± 0.35)	5.594E-05 (± 8.55E-06)	-0.912 (± 0.098)

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

Data comparison α/β-combinations	F-statistic Ouabain	F-statistic Calotropin
αAβ1 – αAβ2	$F_{2,8}$= 0.074, p= 0.929	$F_{2,8}$= 1.319, p= 0.320
αAβ1 – αAβ3	**$F_{2,8}$= 5.218, p= 0.035**	$F_{2,8}$= 0.728, p= 0.512
αAβ2 – αAβ3	**$F_{2,8}$= 6.092, p= 0.025**	$F_{2,8}$= 0.079, p= 0.925
αBβ1 – αBβ2	**$F_{2,8}$= 10.981, p= 0.005**	$F_{2,8}$= 2.607, p= 0.134
αBβ1 – αBβ3	$F_{2,8}$= 3.781, p= 0.070	$F_{2,8}$= 0.724, p= 0.514
αBβ2 – αBβ3	$F_{2,8}$= 0.455, p= 0.650	$F_{2,8}$= 0.655, p= 0.545
αCβ1 – αCβ2	$F_{2,8}$= 0.745, p= 0.505	$F_{2,8}$= 1.887, p= 0.213

αCβ1 – αCβ3	$F_{2,8}$= 1.535, p= 0.273	$F_{2,8}$= 3.402, p= 0.085
αCβ2 – αCβ3	$F_{2,8}$= 1.080, p= 0.384	$F_{2,8}$= 2.217, p= 0.171
αAβ1 – αBβ1	$F_{2,8}$= 1.039, p= 0.397	$F_{2,8}$= 0.554, p= 0.595
αAβ1 – αCβ1	**$F_{2,8}$= 22.729, p< 0.001**	**$F_{2,8}$= 8.724, p= 0.010**
αBβ1 – αCβ1	**$F_{2,8}$= 11.975, p= 0.004**	**$F_{2,8}$= 8.795, p= 0.010**
αAβ2 – αBβ2	**$F_{2,8}$= 57.311, p< 0.001**	**$F_{2,8}$= 9.763, p= 0.007**
αAβ2 – αCβ2	**$F_{2,8}$= 4.835, p= 0.042**	**$F_{2,8}$= 6.422, p= 0.022**
αBβ2 – αCβ2	$F_{2,8}$= 2.353, p= 0.157	**$F_{2,8}$= 13.589, p= 0.003**
αAβ3 – αBβ3	**$F_{2,8}$= 32.590, p< 0.001**	**$F_{2,8}$= 37.553, p< 0.001**
αAβ3 – αCβ3	**$F_{2,8}$= 33.295, p< 0.001**	**$F_{2,8}$= 19.271, p< 0.001**
αBβ3 – αCβ3	$F_{2,8}$= 1.614, p= 0.258	**$F_{2,8}$= 8.239, p= 0.011**

Table 3: Comparison between dose response curves for individual α/β-complexes inhibited by ouabain versus calotropin analyzed with Origin-Pro 9.1G. Significant differences are highlighted in bold.

Data comparison α/β-combinations	F-statistic calotropin vs. ouabain	more potent inhibitor
αAβ1	**$F_{2,8}$= 11.752, p= 0.004**	ouabain
αAβ2	$F_{2,8}$= 2.421, p= 0.151	equal
αAβ3	$F_{2,8}$= 0.589, p= 0.577	equal
αBβ1	$F_{2,8}$= 0.343, p= 0.720	equal
αBβ2	$F_{2,8}$= 4.257, p= 0.055	equal

αBβ3	$F_{2,8}$= 3.054, p= 0.103	equal
αCβ1	**$F_{2,8}$= 35.226, p< 0.001**	calotropin
αCβ2	**$F_{2,8}$= 100.356, p< 0.001**	calotropin
αCβ3	**$F_{2,8}$= 74.665, p< 0.001**	calotropin

226

227 # Discussion

228 Here we have identified the full length coding sequences of three Na,K-ATPase α- and
229 three full length β-subunits of *O. fasciatus* and functionally characterized all nine possible
230 recombinant α/β enzyme complexes. These nine functional enzymes strongly differed in
231 their activity levels. We found that enzyme complexes with αC had a significantly higher
232 activity compared to the other constructs. This perfectly fits with the fact that αC is mainly
233 expressed in the nervous tissue of *O. fasciatus* (11,21) where the Na,K-ATPase plays a
234 crucial role in maintaining electric potentials that guarantee the excitability of neurons.
235 Consequently, a high ionic pumping activity is indispensable. Two of our nine α/β-
236 complexes clearly stand out amongst the other enzyme complexes: αAβ2 and αCβ3. The
237 activity of αAβ2 is reduced in comparison to the other αA and αB constructs that all show
238 similar activity levels. Therefore, we suppose that αAβ2 is not a predominant enzyme *in*
239 *vivo*. In contrast, the activity of αC combined with β3 is significantly higher and would
240 therefore fit well with a natural occurrence of this complex in the nervous tissue. Data of
241 an ongoing proteomic study support this assumption and show that the subunits αC and
242 β3 are most abundant in the nervous tissue of *O. fasciatus* (M. Herbertz et al.,
243 unpublished data).

244 Regardless of the strikingly high overall resistance to inhibition by cardenolides that Na,K-
245 ATPase preparations of *O. fasciatus* display (12,13,26) we found that the individual α/β-
246 combinations strongly differed in their resistance against the two cardenolides ouabain
247 and calotropin. These two cardenolides differ in their structure, steric conformation and
248 by their ability to permeate membranes, as reflected by their lipophilicity. Ouabain is
249 produced in plants of the genus *Acokanthera* and in seeds of *Strophanthus gratus* (both
250 Apocynaceae), it is commercially available, very polar and routinely used in studies on
251 Na,K-ATPase resistance to cardiac glycosides (1,12,23,25,27). Calotropin, on the other
252 hand, is a common compound in plants of the tribe Asclepiadeae (Apocynaceae) to which
253 the host plants of *O. fasciatus* belong. The two cardenolides differ in the conformation of
254 the steroid rings, which is supposed to determine inhibitory potency. The A/B and C/D
255 rings of ouabain, and many other cardenolides, are *cis*-fused and the B/C ring is *trans*-
256 fused (*cis-trans-cis*) (15,31). In contrast calotropin and other cardenolides of *Asclepias*
257 species, have a *trans-trans-cis* conformation (15) that is predicted to lower the toxicity
258 (32) and the binding affinity of the cardenolide (31). This may, however, be compensated
259 by the doubly-linked sugar moiety, typical of *Asclepias* cardenolides (33, 34). A recent
260 study testing various cardiac glycosides against the Na,K-ATPase of the monarch butterfly,
261 *Danaus plexippus,* which is also specialized on *Asclepias* species, actually found calotropin
262 and its isomer calactin to be the strongest tested inhibitors, with calotropin inhibiting the
263 monarch's Na,K-ATPase 60-fold more strongly than ouabain (35).

264 In contrast to the monarch butterfly, there is evidence that the coevolutionary association
265 between the milkweed bugs and their host plants led to lowered sensitivity towards

266 calotropin. While the phylogenetically older αC-subunit (11, 26) was roughly 20-times
267 more strongly inhibited by calotropin than by ouabain not only the overall sensitivity to
268 both cardenolides but also the relative inhibition by calotropin compared to ouabain
269 drastically decreased in the phylogenetically more derived αA- and αB-subunits. Dose-
270 response curves of αB-based enzymes did not significantly differ with respect to inhibition
271 by either cardenolide whereas the αAβ1 complex was even more resistant to calotropin
272 than to ouabain. This argues in favor of a specific adaptation to the host plant compounds
273 over the course of the evolutionary interaction between the milkweed bugs and their host
274 plants.

275 The very pronounced insensitivity of the αA- and αB-subunits of *O. fasciatus* to both
276 cardenolides fits well with their abundant expression in the Malpighian tubules (21) where
277 the enzymes are directly exposed to toxins in the hemolymph and a very efficient
278 reabsorption of cardenolides from the primary urine takes place (36). In contrast, the least
279 sensitive but most active αC is the dominant Na,K-ATPase α-subunit in the nervous tissue
280 where it is protected from intruding cardenolides by the perineurium (blood-brain-barrier)
281 which controls xenobiotica access to the nervous system by a tight network of septate
282 junctions and active efflux carriers (37).

283 A comparison of the present results with our previous studies (26,27) on *O. fasciatus* Na,K-
284 ATPase mimics reveals good concordance regarding our conclusions about enzyme
285 activities. We previously introduced one, two, three or four of the potentially resistance-
286 conferring substitutions into the α-subunit of *D. melanogaster* to mimic the known α1-
287 gene copies of *O. fasciatus* (in this order: αD, αC, αB, αA-mimic; c.f. 10,11) and combined

288 them with two *D. melanogaster* β-subunits, Nrv2.2 and Nrv3 (26,27). The overall behavior
289 of these mimics strongly resembled the present data for the original *O. fasciatus* Na,K-
290 ATPase complexes. Most strikingly, the activity levels of the αA and αB mimics were
291 significantly lower than of the Drosophila wildtype enzyme, the αC or the αD (N122H)
292 mimics. The two Drosophila β-subunits also modulated the activity: the association with
293 Nrv3 strongly reduced the activity of all mimics and the Drosophila wildtype enzyme when
294 compared to those bearing Nrv2.2. In the present study, such a large influence of the β-
295 subunit on ion pumping activity was observed only for the αC-subunit while the effect was
296 less pronounced for the αA and αB-subunits.

297 The β-subunits also strongly modulated the resistance of the enzymes against ouabain,
298 but their effect was specific to the α-subunits they were combined with. A combination
299 with Nrv3 instead of Nrv2.2 previously lead to lowered resistance in the αA- and αB-mimic
300 and increased resistance in the αC-mimic (27). In the present study, resistance changed in
301 the same direction with β1, β2, or β3 for αA (decreasing) and αC (increasing IC_{50}) but also
302 lead to increased IC_{50}-values for αB.

303 The most striking differences, however, appear when comparing the overall ouabain-
304 sensitivity of the original enzyme combinations with the previously constructed mimics
305 (27). While the mimics of the *O. fasciatus* αA-copy (featuring the substitutions
306 Q111T,N122H,F786N,T797A) were by far the most insensitive constructs followed by the
307 αB-mimics (with Q111T,N122H,F786N), and the more sensitive αC-mimics (N122H,F786S),
308 the original αA-constructs tested here are at least five times less sensitive than αB-

309 constructs. On the other hand, the original αC-constructs are 10-times more resistant than
310 predicted from the experiments with the αC-mimics.

311 These differences in sensitivity between mimics and original enzymes strongly point to the
312 importance of additional substitutions in the *O. fasciatus* Na,K-ATPase α1-paralogs. When
313 constructing the mimics, we focused on the four most conspicuous amino acid exchanges
314 in the binding pocket of the Na,K-ATPase that were previously shown functionally to be
315 essential for cardiac glycoside binding (25,38,39). However, there are additional amino
316 acid substitutions in the cardiac glycoside binding pocket of the Na,K-ATPase α1-genes of
317 *O. fasciatus* that may further increase their resistance (11,19). These and further
318 additional amino acid exchanges that at first appear inconsequential may be of
319 importance in modulating the effects of the resistance conferring substitutions or
320 ameliorating their pleiotropic effects (28-30).

321 Conclusion

322 Resistance to specific cardiac glycosides, enzyme activity and the emerging knowledge on
323 tissue distribution of the nine α/β-complexes of the large milkweed bug investigated here
324 further support a scenario of stepwise adaptation to the bugs' toxic host plants: The
325 duplications leading to three paralogs of the α1-subunit gene that can be combined with
326 three functional β-subunits enabled a functional diversification of the resulting Na,K-
327 ATPases allowing for tissue specific adaptations. The high activity of the αC/β-complexes is
328 suited for efficient ion transport necessary for excitability of nervous tissue but trades off
329 against lower resistance to high concentrations of cardiac glycosides. These

330 phylogenetically older complexes were relatively more susceptible to the host plant toxin
331 calotropin while the younger αA- and αB-subunits were overall more resistant and
332 relatively less sensitive to calotropin and thus suited for expression in toxin exposed
333 tissues like the Malpighian tubules. These physiological details provide new fascinating
334 insights into the continued escalation of defense and counter-defenses between insects
335 and their poisonous host plants.

336

337

338 # Material and methods

339 **Identification of sequences**

340 Two of the three previously published gene copies of the Na,K-ATPase α1 gene (11) (α1B
341 Acc.No. JQ771519, α1C Acc.No. JQ771518) were incomplete at the 3' end. Both missing
342 ends could be recovered by RACE PCR (SMARTer RACE kit, Takara Bio, Mountain View, CA,
343 USA).

344 Previously published transcriptomic data of *O. fasciatus* (11) were used to identify the full-
345 length coding sequences of the Na,K-ATPase β-subunits. The raw data were trimmed and
346 assembled with Trinity (40) as described in Meinzer et al. (41). *O. fasciatus* β-subunit
347 sequences were recovered using published orthologous sequences from *Locusta*
348 *migratoria* (Orthoptera; Acc.No. KF813098-KF813100) and *Blatta germanica* (Blattodea;
349 Acc.No. HE819405) in tblastn searches against the transcriptome. *De novo* assembly of the

350 raw reads and quality control was performed with the program CLC-Genomics Workbench
351 (Qiagen, Hilden, Germany). The resulting *contigs* were screened for open reading frames
352 (ORFs) with a length of roughly 900 bp (the consensus length of the conserved Na,K-
353 ATPase β-subunit) using the program VectorNTI (Thermo Fisher, Darmstadt, Germany).
354 Homology of the *contigs* were assigned by a further round of tblastx searches against
355 published sequences in genbank.

356 **Construction of recombinant baculoviruses**

357 The coding sequences of the Na,K-ATPase α- and β-subunits genes (α1A, α1B, α1C, β1, β2
358 and β3) were amplified from cDNA transcribed with Superscript III (Thermo Fisher) from
359 RNA extracted from nervous tissue, gut, testes or muscles with the RNAmagic-kit (Bio-
360 Budget Technologies GmbH, Krefeld, Germany). For downstream cloning all subunits were
361 amplified with the Pfu-Phusion Polymerase (Thermo Scientific) and primers including the
362 full coding sequence and incorporating suitable restriction sites at the 5' end.

363 PCR products were restricted with the chosen enzymes (Thermo Fisher), gel extracted
364 (QIAquick gel extraction kit, Qiagen) and ligated into pFastBac-Dual vectors (Thermo
365 Fisher). The final plasmids had an α-subunit under the control of the polyhedrin promoter
366 (PH) and a β-subunit under the p10 promoter. Recombinant baculoviruses were generated
367 as previously described (27) and transfected into *Sf*9 cells to obtain a first (P1) and second
368 (P2) generation of recombinant virus stock. Uninfected *Sf*9 cells have no or negligible
369 Na,K-ATPase abundances so that contaminating endogenous Na,K-ATPase activities can be
370 excluded (42).

371 **Production of recombinant proteins**

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

380 **SDS-PAGE/ Western blotting**

381 Specific binding of customized β-specific anitbodies (Davids Biotechnology, Regensburg,
382 Germany) was additionally validated in our lab by applying the antibodies on two
383 experimental setups. First, the specificity of the antibodies was tested with heterologously
384 expressed *O. fasciatus* Na,K-ATPase protein and second, with four different tissue extracts
385 (muscles, Malpighian tubules, ovaries and nervous tissue). The α1/β1, α1/β2, α1/β3, and
386 α1/βx cell protein construct extracts (4x 30 µg) and muscles, Malpighian tubules, ovaries,
387 and nervous tissue extract (4x 35 µg for each tissue) were run on a SDS-PAGE followed by
388 western blotting as described below. All four Na,K-ATPase constructs and tissues were
389 immunostained with each of the specific β-antibodies. These stainings verified that no
390 unspecific binding occurs (Fig. S2 & S3), but that the β-specific antibodies solely detect
391 their target-subunit in all tested samples.

392

393 Thirty µg of the heterologously expressed proteins were deglycosylated with
394 endoglycosidase H following the manufacturer's protocol (Promega GmbH, Wiesloch,
395 Baden Württemberg, Germany) but instead of boiling proteins for 5 minutes at 95°C we
396 performed the denaturation step at 50°C for 30 minutes to maintain the integrity of the
397 protein. SDS-polyacrylamide gel electrophoresis and western blotting was performed as
398 previously reported (27) with few modifications. The membrane proteins were blotted on
399 a nitrocellulose membrane for 90 minutes at 100 V with an additional ice block at 4°C. To
400 mask unspecific binding sites the membrane was blocked with BlueBlock PF (Serva,
401 Heidelberg, Germany). The split membranes were incubated with the primary monoclonal
402 antibodies α5, 12G10 anti-α-tubulin (DSHB Hybridoma Bank, University of Iowa; deposited
403 by D.M. Fambrough (43) and by J. Frankel and E.M. Nelsen), and customized specific
404 polyclonal antibodies for the subunits β1, β2, and β3 (Davids Biotechnology, Regensburg,
405 Germany). For detection of the primary antibodies the membrane was incubated for one
406 hour with goat anti-mouse (α5, 12G10 anti-α-tubulin), anti-rabbit (β2), and anti-chicken
407 (β1, β3) secondary antibodies conjugated with horseradish peroxidase (Dianova,
408 Hamburg, Germany) and developed with 4-chloro-1-naphtol as previously described (27).

409

410 **Extraction and preparation of cardenolides**

411 Calotropin was extracted according to (35) from approximately 8 g of dried larvae of the
412 monarch butterfly *Danaus plexippus* reared on *Asclepias curassavica*. The cardenolides
413 were extracted with methanol, lipids extracted with hexane, and the cardenolides

414 retrieved by chloroform extraction. The concentrated methanolic extract was subjected to
415 preparative HPLC (Agilent 1100) equipped with a photodiode array detector and a semi-
416 preparative RP-Nucleodur C18 Htec column (5 µm, 250 x 10 mm, Macherey-Nagel, Düren,
417 Germany) using a water-acetonitrile gradient. Calotropin was identified by its retention
418 time and checked for molecular weight and LS-MS spectrum by LC-ESI-TOF spectrometry
419 by Dr. Maria Riedner (Department of Chemistry, Universität Hamburg) and the structure
420 confirmed by NMR spectroscopy. The resulting calotropin preparation was 80% pure.

421 Calotropin and the commercially available ouabain (O3125, Sigma-Aldrich) were first
422 dissolved in DMSO and prepared for Na,K-ATPase activity assays as dilutions in sterile H_2O
423 reaching final concentrations from 10^{-8} to 10^{-3} M with 2% DMSO.

424

425 **Na,K-ATPase activity and inhibition assay**

426 Activities of the nine different Na,K-ATPase α/β-subunit combinations of *O. fasciatus* were
427 determined by photometric measurements of inorganic phosphate released from
428 enzymatic ATP hydrolysis while subtracting background ATPase activity following
429 previously described methods (27,44). Based on previous results (27) the buffer regime
430 used was 50 mM NaCl, 4 mM $MgCl_2$, 50 mM imidazol, 20 mM KCl, pH 7.4. The inhibition of
431 the different Na,K-ATPases was tested under increasing ouabain and calotropin
432 concentrations and expressed as percentage activity of a non-inhibited control. For each
433 α/β-subunit construct technical duplicates of three biological replicates of recombinant
434 proteins isolated from independently transfected *Sf*9 cells were tested.

435

436 **Statistic analysis of data**

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

449

450 ## Declarations

451 **Ethics approval and consent to participate**

452 Not applicable.

453 **Consent for publication**

454 Not applicable.

455

456 **Availability of data and materials**

457 The datasets generated during the current study are available in the Dryad repository at

458 https://doi.org/10.5061/dryad.j0zpc86dg, sequence data will be deposited in the European

459 Nucleotide Archive (ENA) upon acceptance.

460 **Competing interests**

461 The authors declare that they have no competing interests.

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466 role in the design of the study and collection, analysis, and interpretation of data and in

467 writing the manuscript.

468

469 **Author's contribution**

470 SDa and SDo conceptualization; SDa, MW, VW, RT, MH cloning and protein expression;

471 SDa and MW enzyme assays, data analysis and visualization; MW and SDo writing-original

472 draft; SDa writing-review and editing.

473

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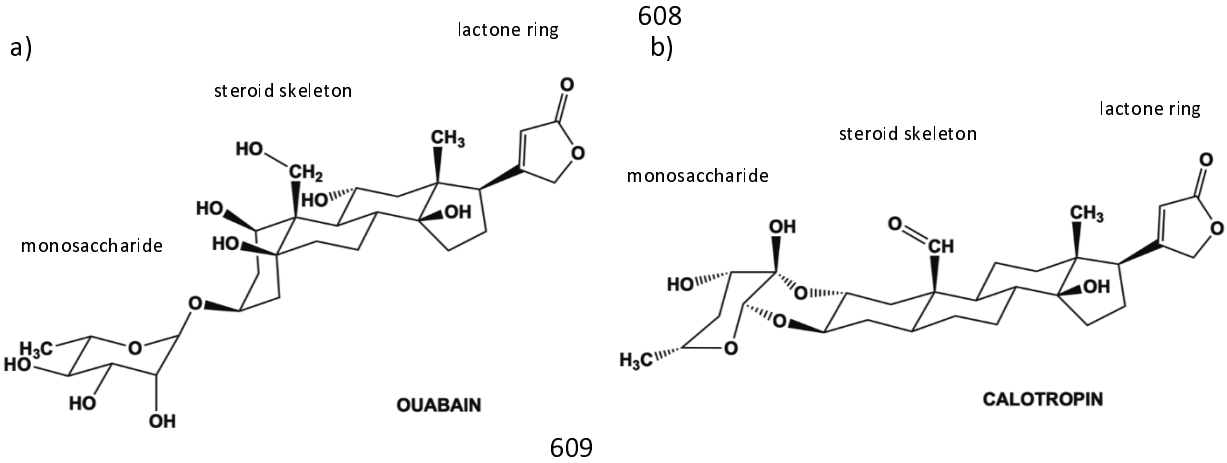


Figure 1: Structure of the cardenolides ouabain (a) and calotropin (b). Both are characterized by a monosaccharide attached to a steroid skeleton at C3 (4 steroid rings: A-D) and a five-membered unsaturated lactone ring at C-17. a) In ouabain the monosaccharide is attached by a single glycosidic link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

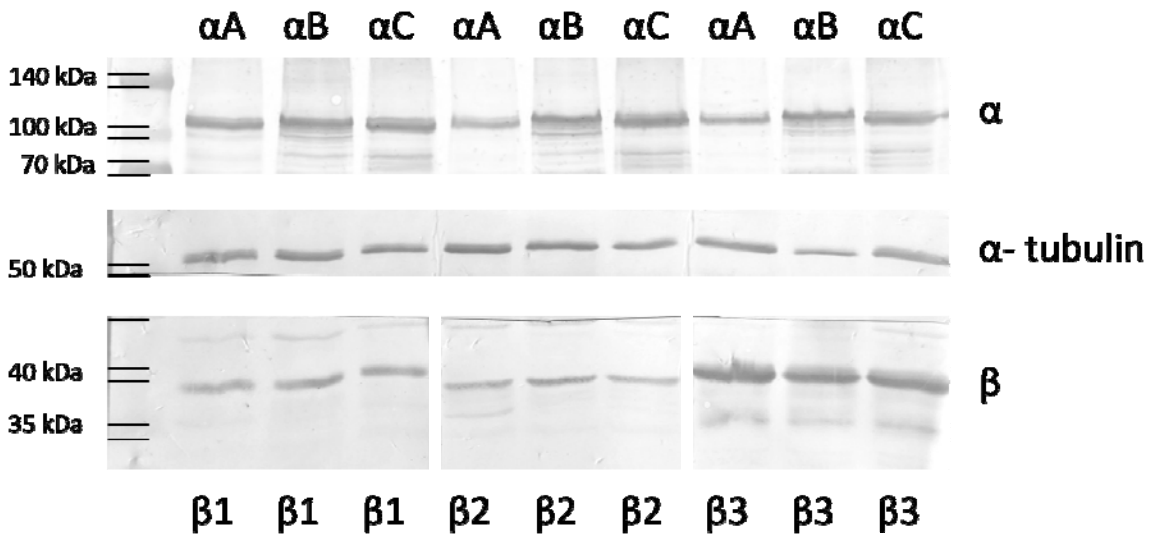


Figure 2: Western blot of nine α/β-complexes of *O. fasciatus* expressed heterologously in cell culture. 30 µg of protein were separated by SDS-PAGE and blotted on a nitrocellulose membrane. The upper membrane slice shows at a size of 110 kDa αA, αB and αC in alternating order, the proteins were stained with the primary antibody α5 (DSHB hybridoma bank). At 55 kDa α-tubulin is shown as a positive control (middle membrane), the protein is stained with the primary antibody 12G10 anti-α-tubulin (DSHB hybridoma bank). Primary antibodies of both proteins were detected with the secondary HRP-conjugated antibody goat-anti mouse (Dianova). The associated β-subunits 1, 2, and 3 were deglycosylated in advance. The lower membrane is split into three slices, the first one shows β1, the second β2, and the third β3. The subunits were stained with β-subunit specific customized primary antibodies (produced by Davids Biotechnology) and HRP-conjugated secondary antibodies goat-anti rabbit (for β2, Dianonva) and goat-anti chicken (for β1 and 3, Dianova). The different β-subunits show bands at weights ranging between 35 and 40 kDa. The bands were visualized with 4-CN. Please note: Signal strength is not comparable between the different β-subunits, due to different binding affinities of the antibodies. Due to the grey scale some bands of the multicolor protein ladder were marked by lines.

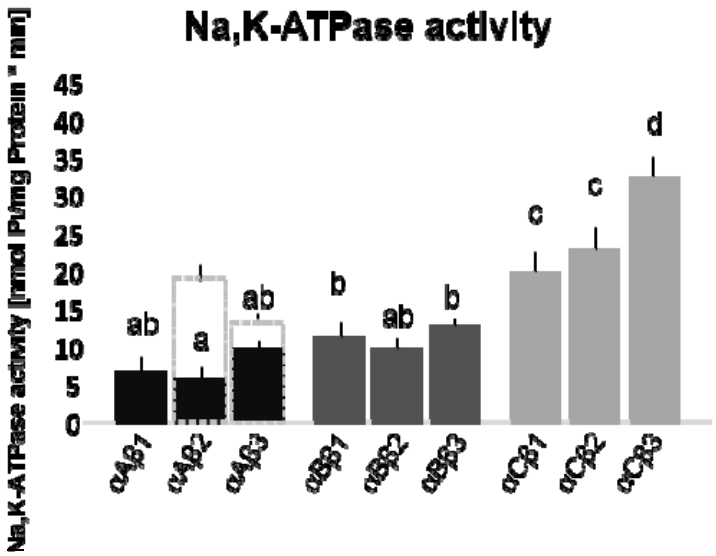
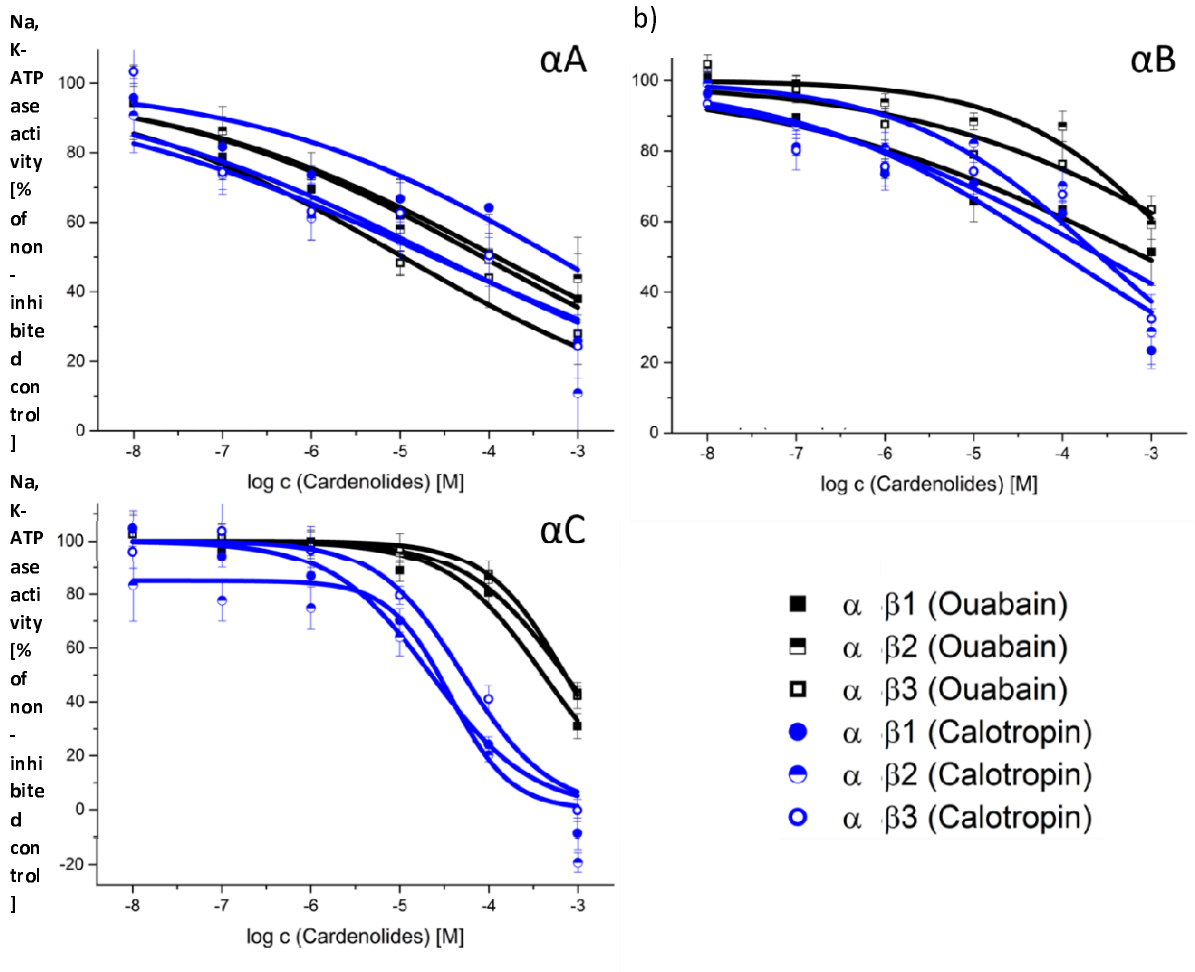


Figure 3: Na,K-ATPase activity [nmol Pi/mg protein*min] of nine α/β-complexes of *O. fasciatus.* Each bar depicts the mean and standard deviation of three biological replicates. Different letters above the bars indicate significant differences between enzymes (ANOVA, $F_{8,18}$=77.97, p<0.001). Black bars: αA with three β-subunits and overall activities ranging from **5.5 to 9.3 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 (Dalla et al., 2017). Dark gray bars: αB/β-complexes ranging from **10.3 to 13 nmol Pi/mg protein*min**. Light gray bars: αC/β-complexes with activity levels ranging from **19.7 to 32.7 nmol Pi/mg protein*min**. The activity levels of the three αC/β-enzymes are significantly higher than those of the other enzymes, with the highest activity reached by αC combined with β3.



649

650 **Figure 4: *In vitro* inhibition by calotropin and ouabain of nine α/β-complexes of *O.***
651 ***fasciatus* expressed heterologously in cell culture. Dose response relationships between**
652 **increasing cardenolide concentrations and residual enzyme activities were expressed in**
653 **% of the non-inhibited control (calculated with Origin Pro 9.1G).** Each data point
654 represents the mean of three biological replicates and standard deviation. Effect of
655 calotropin (blue curves and round symbols) and ouabain (black curves and square
656 symbols) a) on αA, b) on αB, and c) on αC combined each with β1, 2, and 3. Please note
657 the different scales.

658

659

Supplemental

```
Of_ß1 MKGKTGNH-KEAEYSWGYKKPEDDNRTLKEKIRIAFYNPSTGTVLGRTPLDWGRLLLFYILLYLALALLFAICMKGL
Of_ß2 MEANTQEKKKEVGF---YSAPK--QLTGWAYIQNFLYDPVEGKVLGRTGPSWAKIGVFYLIFYSVLASLFGIMLWIF
Of_ß3 MADKAKEE-----F---YTRPA--KVGNWDAFKLFLWNSETSQFLGRTFSSWAKILLFYLIFYACLMGFFSALLALF
Of_ßx ------------------------------------------------------------------MKHLLAVLFCFL

Of_ß1 LLTIDLKHPKWILEESIIGTNPGMGYRPMPKMAEDGKSLVWYQANNKSNVAMWEESINEFLKPYMNEH---EFTNRM
Of_ß2 YHTLDPRVPRWQLRESLIGQDPGLGFRPMPNDTDSRSTLLWYQGKSRESCNLWTKTLENFLEVYRKPGLTPGRGQNI
Of_ß3 FQTLDFKQPKWQQASSLIGSNPGLGFRPMPPESHVESTLIWFKV-SESNYATWTNKLDEFLKPYREPD--PKYENVM
Of_ßx QHSL--------------GDQITLKVHPLSEDS--KSTLIWF---NSSTYDRWAQQLDNFLEVYRNPTLTDGREENL

Of_ß1 F-CDYDMPKPSEDKVCEVYINETSWGPCTKRNHYSFPSSSPCVFLKLNRIYGWTPEYYEDLSSLPPEMPSNLRSFI
Of_ß2 YNCDYDRP-PGKGQVCDVDVKS--WYPCTLENNFNYHLSSPCVFIKLNKIYGWQPEYYNDSRDFPETMPEDLKNHI
Of_ß3 QSCDFDTP-DTPDRVCKVDIKS--WSPCVKENNYAYHNGAPCIFLKLNKIYGWMPEFYNDTETLPKDMPEDLKSHI
Of_ßx VTCNYNKY-PSTDSVCNIRPEE--MKPCTASNFFGYKSGTPCVFIELEKVLGWKPKYFSSGKSLPKTMPKNLKEKI

Of_ß1 ENEKAIDPRRMNTIWVSCNPETPFDNETMGEVEYYPRQGFPGYYYPYKNQDGYLSPLIAVRFKRPPVGILVNVECR
Of_ß2 LTQKAINPELIKTIWVSCEGESPHDRENIGEIQYIPRQGFPGYFYPYVNTEGYLSPLVAVNFKRPKTGVIINVECR
Of_ß3 NEEKKHNPKSLNMVWVSCEGENPADVENIGSIQYIPRRGFPGFYFPYKNQEGYLSPVIAIWFEKPVNGILINIECK
Of_ßx LETTEVNLKMWETIWLSCDGETPADKEMIGPIQILPQPGFPGYFFPYIGQPGYLNPVVAVHFERPLGGLVINIVCK

Of_ß1 AWAKNIIYQRPTGAKDRQGSVHFEILVDP*
Of_ß2 AWAKNLKVDR----KERIGIVHFELLID*
Of_ß3 AWARNIHHDR----AERRGSVHFELMID*
Of_ßx AWAPGIIHDM----NKPLGVARFELLID*
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Figure S1: Alignment of deduced *O. fasciatus* Na,K-ATPase α and β-subunit protein sequences. Full length coding sequence of all four β-subunits, tyrosine rich motif typical for β-subunits in orange, cysteine residues forming three conserved intramolecular disulfide bridges in yellow, potential glycosylation sites in blue, and peptides used to raise β-subunit specific antibodies are marked in grey. The N-terminal truncated βx was not further investigated in this study.

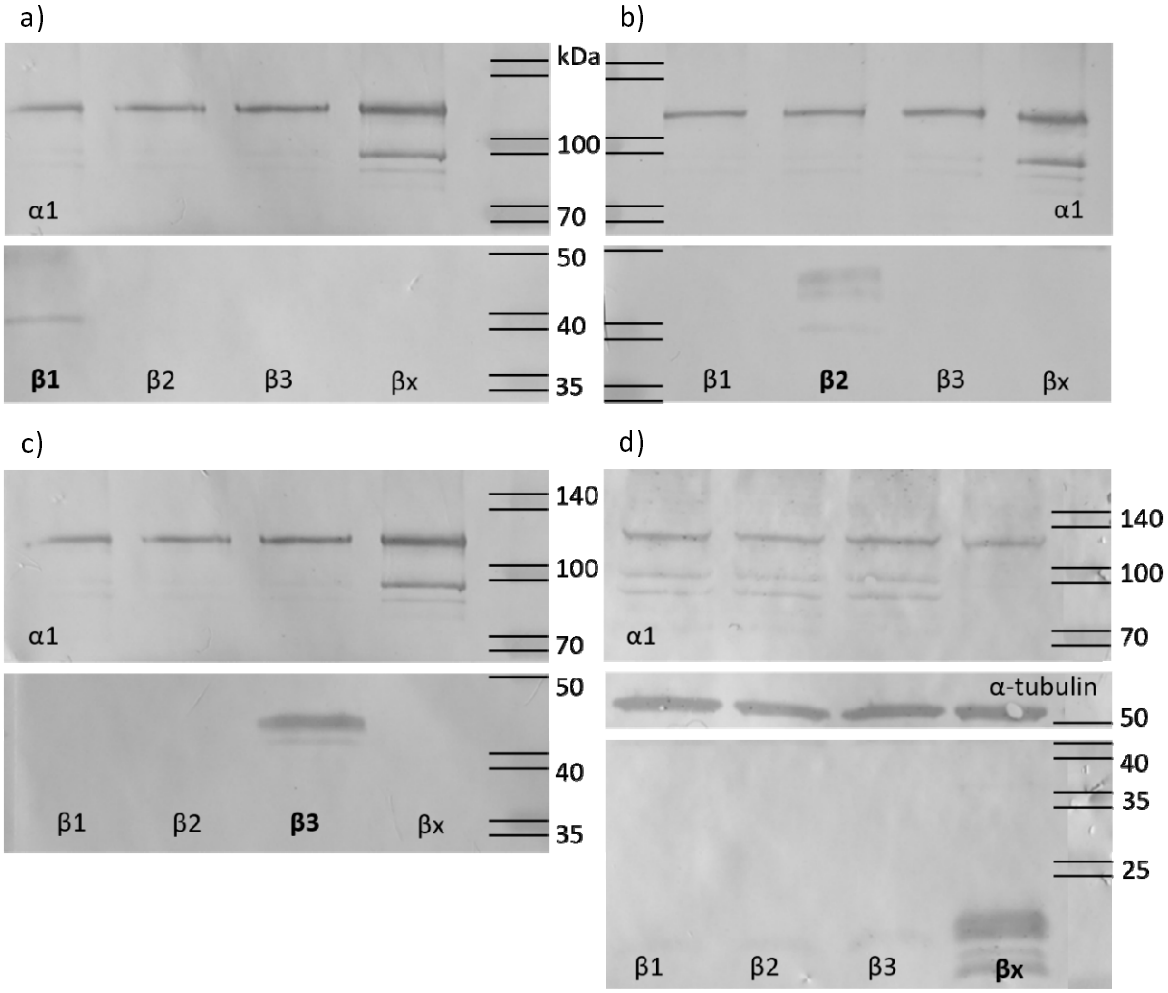
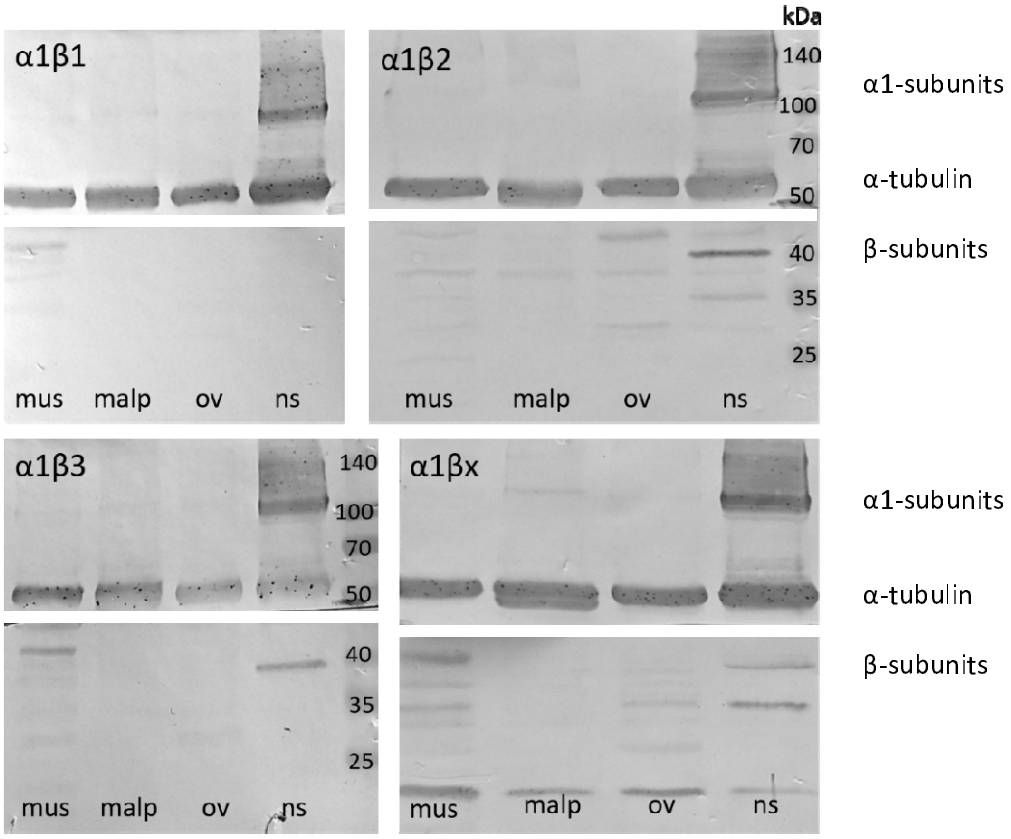


Figure S2: Antibody specificity test with 30µg of heterologously expressed α/β enzymes via SDS-PAGE and western blot. Each set of α1 combined with β1, β2, β3, and βx respectively was stained with α5 primary antibody (upper membrane with α1-subunits at 110 kDa) and one of the four β-specific antibodies: a) β1, b) β2, c) β3, and d) βx-specific antibody, lower membrane with dissociated β-subunits). The middle membrane of the last set (d) was additionally stained with 12G10-anti-α-tubulin. Both, the α-tubulin and α1-staining function as a positive control. The primary antibodies were detected with HRP-conjugated secondary antibody (α5 and 12G10-anti-α-tubulin: goat-anti mouse, β1 and β3: goat-anti chicken, β2 and βx: goat-anti rabbit). The western blots show that the β-specific antibodies solely stain their target subunits (bold letters mark the specific stained β-subunits).



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685 **Figure S3: Antibody specificity test with 35µg four different tissue extracts (muscle**
686 **(mus), Malpighian tubules (malp), ovaries (ov), nervous tissue (ns)) via SDS-PAGE and**
687 **western blot.** On each membrane set, consisting of an upper and lower slice, one α1/β-
688 complex is shown. Each set of four different tissue extracts was stained with α5 primary
689 antibody (α1: 110 kDa) and 12G10-anti-α-tubulin (α-tubulin: 55 kDa) on the upper slices
690 and one of the four β-specific antibodies (β: 30-45 kDa; from upper row left to bottom
691 row right: β1-, β2-, β3- and βx-specific antibody) on the lower slices. The 12G10-anti-α-
692 tubulin staining functions as a positive control. The primary antibodies were detected with
693 HRP-conjugated secondary antibody (α5 and 12G10-anti-α-tubulin: goat-anti mouse, β1
694 and β3: goat-anti chicken, β2 and βx: goat-anti rabbit). The immunostained western blots
695 show differences between abundances and glycosylation pattern in each tissue unique for
696 each β-subunit.

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