Community-wide convergent evolution in insect adaptation to toxic cardenolides by substitutions in the Na,K-ATPase

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Edited by May R. Berenbaum, University of Illinois at Urbana–Champaign, Urbana, IL, and approved June 19, 2012 (received for review February 5, 2012)

The extent of convergent molecular evolution is largely unknown, yet is critical to understanding the genetics of adaptation. Target site insensitivity to cardenolides is a prime candidate for studying molecular convergence because herbivores in six orders of insects have specialized on these plant poisons, which gain their toxicity by blocking an essential transmembrane carrier, the sodium pump (Na,K-ATPase). We investigated gene sequences of the Na,K-ATPase α-subunit in 18 insects feeding on cardenolide-containing plants (spanning 15 genera and four orders) to screen for amino acid substitutions that might lower sensitivity to cardenolides. The replacement N122H that was previously shown to confer resistance in the monarch butterfly (*Danaus plexippus*) and *Chrysochus* leaf beetles was found in four additional species, *Oncopeltus fasciatus* and *Lygaeus kalmii* (Heteroptera, Lygaeidae), *Labidomera clivicollis* (Coleoptera, Chrysomelidae), and *Liriomyza asclepiadis* (Diptera, Agromyzidae). Thus, across 300 Myr of insect divergence, specialization on cardenolide-containing plants resulted in molecular convergence for an adaptation likely involved in coevolution. Our screen revealed a number of other substitutions connected to cardenolide binding in mammals. We confirmed that some of the particular substitutions provide resistance to cardenolides by introducing five distinct constructs of the *Drosophila melanogaster* gene into susceptible eucaryotic cells under an ouabain selection regime. These functional assays demonstrate that combined substitutions of Q^{111} and N^{122} are synergistic, with greater than two-fold higher resistance than either substitution alone and >12-fold resistance over the wild type. Thus, even across deep phylogenetic branches, evolutionary degrees of freedom seem to be limited by physiological constraints, such that the same molecular substitutions confer adaptation.

insect–plant interactions | specialist herbivores | target site insensitivity | toxin resistance | cardiac glycosides

The extent to which adaptive evolution is predictable at the molecular level is still highly debated (1, 2). When evolution proceeds by alterations in genes of major effect, convergence at the molecular level is more likely than if many genes are involved (3). Nonetheless, the genetic basis of adaptation is known only from a handful of organisms. In host–parasite coevolution, which is thought to be a widespread evolutionary interaction, gene-for-gene interactions tend to follow cycles of predicable change in both animal and plant systems (4, 5). However, in most plant–herbivore interactions, our understanding of the genetics of plant defense has far outpaced our understanding of insect adaptation to these defenses (6, 7). To address the convergence of molecular adaptation, here we examine a diverse community of insect herbivores (18 species, representing deep phylogenetic divergences; Fig. 1) specialized on plants that produce cardendolides (cardiac glycosides), a class of potent toxins that block Na,K-ATPase.

The Na,K-ATPase, or sodium pump, is a transmembrane ion-motive enzyme and the most important ion carrier in animal tissues responsible for the maintenance of membrane potentials (8–10). The structure and amino acid sequence of the Na,K-ATPase is highly conserved among animals: the catalytic α-subunit has 10 transmembrane domains and five extracellular loops (9, 11). By binding to Na,K-ATPase, cardenolides make remarkably general animal toxins that function in a dose-dependent manner. Because of their common 23 C four-ring steroid skeleton with a five-membered lactone ring at C17, all cardenolides fit from the extracellular side into a binding pocket of the Na,K-ATPase, whereas the glycosidically bound sugars of varying number and structures face toward the surface. A number of amino acids were shown to be involved in cardenolide binding using ouabain, the most widely used reference cardenolide from *Strophantus gratus* (Apocynaceae) (9, 11–17). Although sensitivity of the Na,K-ATPase to cardenolides is a prevalent characteristic in the majority of animals, a number of insect herbivores have adapted to cardenolide-containing plants (18).

In the monarch butterfly, *Danaus plexippus*, and the large milkweed bug, *Oncopeltus fasciatus*, in vitro assays showed that these species possess Na,K-ATPases that have a dramatically lowered binding affinity to cardenolides (ouabain) (19–22). Molecular investigations later demonstrated that this insensitivity may be explained in the monarch butterfly, at least in part, by an amino acid substitution of asparagine for histidine at position 122 (N122H) in the first extracellular loop of the Na,K-ATPase (19, 23). In vitro expression of the *Drosophila melanogaster* Na,K-ATPase α-subunit with the N122H replacement transfected into HEK cells resulted in increased resistance to ouabain (19). However, apart from the monarch butterfly, this resistance-conferring substitution has thus far only been detected in *Chrysochus* leaf beetles (24), which also feed on cardenolide-containing Apocynaceae. Several other lepidopterans that typically feed on cardenolide-containing plants, including some congeners of the monarch butterfly, do not contain this substitution (19, 25, 26). Thus, there may be a diversity of strategies in how insects specialize on cardenolide-containing plants, and the broad extent of molecular convergence is unclear.

Here we directly address the prevalence of resistance-conferring amino acid substitutions by genetically screening the Na,K-ATPase of 18 species in four insect orders (Coleoptera, Lepidoptera, Diptera, and Heteroptera), which are specialist herbivores of cardenolide-containing *Asclepias* or *Apocynum* species. To increase the number of available independent phylogenetic comparisons, we additionally sequenced some nonadapted relatives.

Author contributions: S. Dobler and A.A.A. designed research; S. Dalla and V.W. performed research; S. Dobler analyzed data; and S. Dobler and A.A.A. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The sequences reported in this paper have been deposited in the European Molecular Biology Laboratory Nucleotide Sequence database, www.ebi.ac.uk/embl (accession nos. HE956736 to HE956756).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1202111109/-/DCSupplemental.

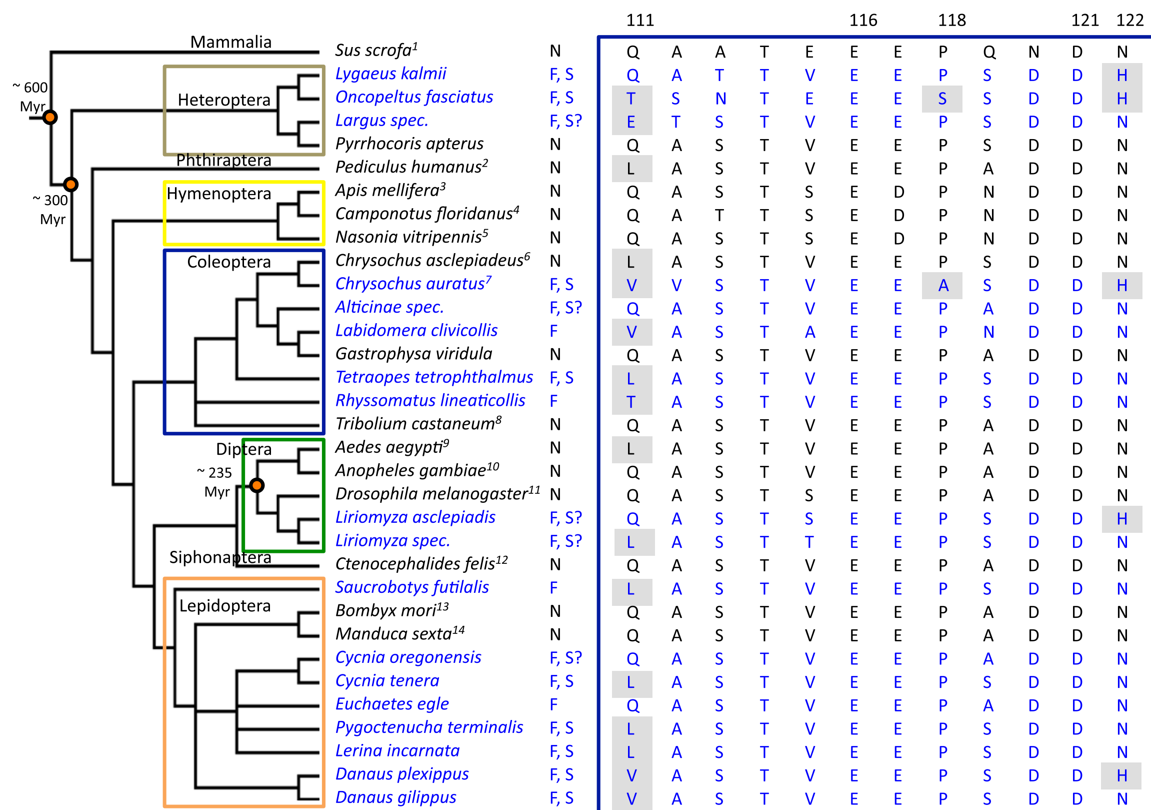


Fig. 1. Comparison of the first extracellular loop of the Na,K-ATPase α-subunit of insects across their phylogeny (compiled from refs. 50 and 51, calibration points according to ref. 52). Species typically encountering cardenolides in their hosts are in blue, those not exposed are in black. The known adaptations to cardenolides are indicated next to the species names: N, noncardenolide feeder; F, feeds on cardenolide plants; S, sequesters cardenolides; S?, sequestration unknown. Numbers of amino acids previously implied in the mammalian enzyme to be connected to ouabain binding are given, numbering corresponds to the mature pig enzyme (*Sus scrofa*). Amino acid residues shaded in gray potentially alter cardenolide sensitivity. A, alanine; D, aspartic acid; E, glutamic acid; H, histidine; L, leucine; N, asparagine; P, proline; Q, glutamine; S, serine; T, threonine; V, valine. GenBank accession nos.: [1]X03938, [2]XM_002427669, [3]XM_003250374, [4]EFN66583, [5]XR_134120, [6]AJ617742, [7]AJ617743, [8]XM_969867, [9]XM_001662166, [10]XM_003436221,[11]AF044974, [12]S66043, [13]BGIBMGA005058 (Silkworm Genome Database, http://silkworm.genomics.org.cn/silkdb/), [14]S51591.

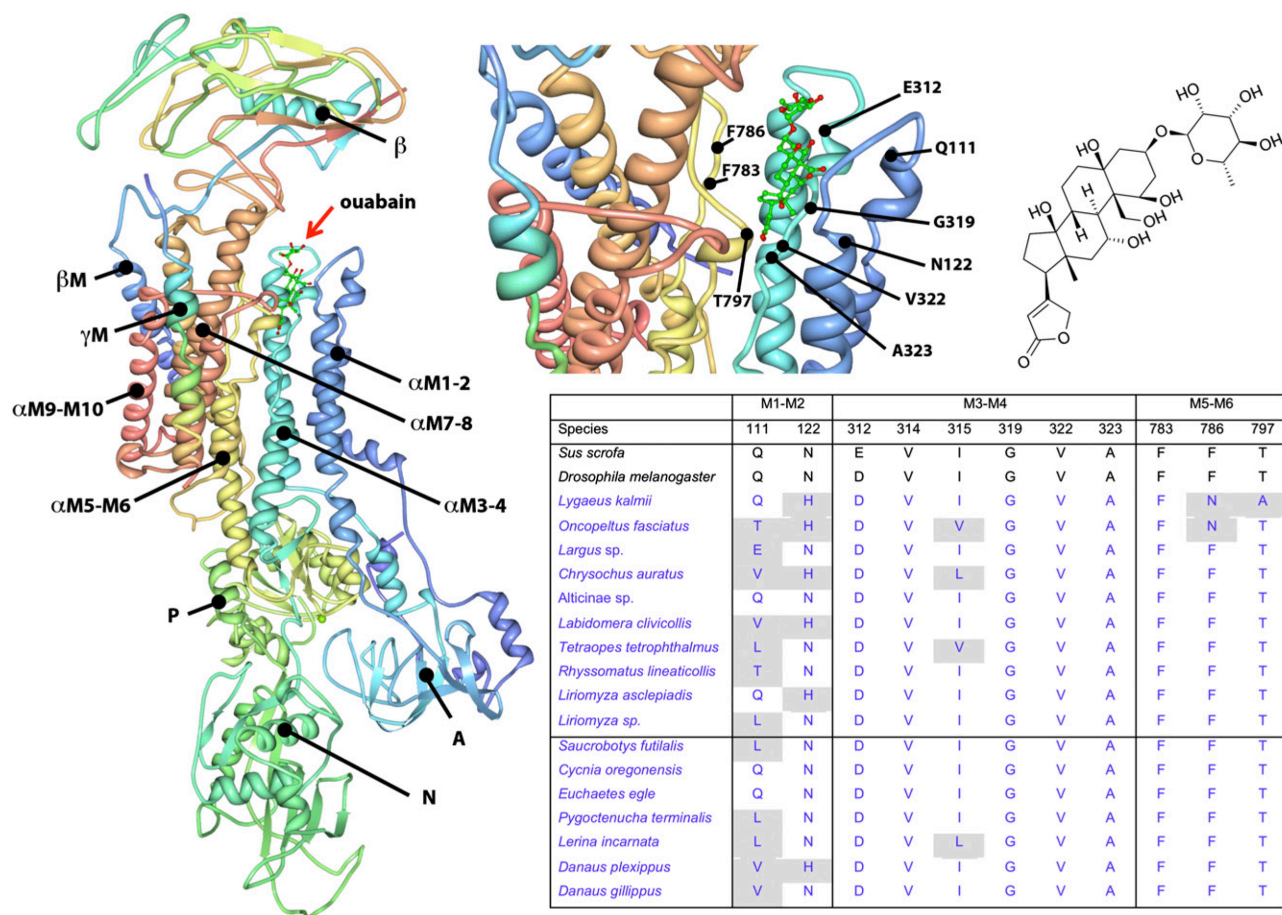
We specifically focused on substitutions at amino acid positions connected with sensitivity to cardenolides in the mammalian enzyme. Because we found a small set of highly convergent molecular substitutions, we functionally tested whether five of these mutations (and a combination of two) confer reduced sensitivity to cardenolides using the susceptible gene of *D. melanogaster* in a growth and survival assay of transfected cells.

Results

Na,K-ATPase α-subunit sequences of the homologous gene to the functional copy of the fruit fly [AF044974 (27)] were recovered as overlapping fragments covering a stretch from the first to the sixth transmembrane domain (amino acids 95–805 of the mature pig enzyme) for 17 of the 18 insect species living on cardenolide-containing North American Apocynaceae (i.e., all but *Cycnia tenera*, see below) and three noncardenolide adapted relatives (*Chrysochus asclepiadeus*, *Gastrophysa viridula*, and *Pyrrhocoris apterus*). This gene region is highly conserved, with a minimum of 78% amino acid identity between the vertebrate and insect sequences (i.e., a maximal p-distance of 0.221) and a minimum of 87% amino acid identity among the insect sequences (for species in Fig. 2). Synonymous substitutions, on the other hand, had reached saturation, with a proportion of 0.942 between pig and insects and of 0.965 among insects (Table 1).

Substitutions at several residues in the first extracellular loop of the Na,K-ATPase were connected to altered cardenolide sensitivity in the rat and the sheep (reviewed in ref. 28). For four of these (positions 116, 118, 121, and 122, numbering according to the mature pig enzyme), all insects surveyed that do not typically encounter cardenolides bear the same amino acids as the sensitive mammalian enzyme (sheep, pigs, humans, etc.) (Fig. 1). Position 111, which in conjunction with exchanges at position 122 is of prime importance for cardenolide insensitivity in the mammalian enzyme (12, 14), is not as highly conserved. For example, position 111 bears a leucine instead of a glutamine residue in the mosquito *Aedes aegypti*, the body louse *Pediculus humanus corporis*, and the leaf beetle *C. asclepiadeus*.

In our survey of 18 cardenolide-adapted species, we found four additional incidences of the N122H replacement previously reported for *D. plexippus* and *Chrysochus auratus* (19, 24): the lygaeid bugs *O. fasciatus* and *Lygaeus kalmii*, the chrysomelid beetle *Labidomera clivicollis*, and the agromyzid fly *Liriomyza asclepiadis*. All other insects had the conserved N^{122} at this position (Fig. 1). Although we did not observe any replacements of the conserved amino acids at positions 116 and 121, remarkably, we found that 11 of the 18 cardenolide-adapted herbivores had a substitution at position 111 (Fig. 1). At position 118, where substitutions have also been shown to alter the sensitivity to ouabain (29), the leaf beetle *C. auratus* and the lygaeid bug *O. fasciatus* bear an alanine and serine, respectively. Thus, across roughly 300 Myr of divergence between bugs and holometabolous insects, we find identical amino acid replacements in all four insect orders feeding on cardenolide-containing plants (Fig. 1).



	M1-M2		M3-M4						M5-M6		
Species	111	122	312	314	315	319	322	323	783	786	797
Sus scrofa	Q	N	E	V	I	G	V	A	F	F	T
Drosophila melanogaster	Q	N	D	V	I	G	V	A	F	F	T
Lygaeus kalmii	Q	H	D	V	I	G	V	A	F	N	A
Oncopeltus fasciatus	T	H	D	V	V	G	V	A	F	N	T
Largus sp.	E	N	D	V	I	G	V	A	F	F	T
Chrysochus auratus	V	H	D	V	L	G	V	A	F	F	T
Alticinae sp.	Q	N	D	V	I	G	V	A	F	F	T
Labidomera clivicollis	V	H	D	V	I	G	V	A	F	F	T
Tetraopes tetrophthalmus	L	N	D	V	V	G	V	A	F	F	T
Rhyssomatus lineaticollis	T	N	D	V	I	G	V	A	F	F	T
Liriomyza asclepiadis	Q	H	D	V	I	G	V	A	F	F	T
Liriomyza sp.	L	N	D	V	I	G	V	A	F	F	T
Saucrobotys futilalis	L	N	D	V	I	G	V	A	F	F	T
Cycnia oregonensis	Q	N	D	V	I	G	V	A	F	F	T
Euchaetes egle	Q	N	D	V	I	G	V	A	F	F	T
Pygoctenucha terminalis	L	N	D	V	I	G	V	A	F	F	T
Lerina incarnata	L	N	D	V	L	G	V	A	F	F	T
Danaus plexippus	V	H	D	V	I	G	V	A	F	F	T
Danaus gillippus	V	N	D	V	I	G	V	A	F	F	T

Fig. 2. Amino acid exchanges that may alter cardenolide sensitivity of the Na,K-ATPase of cardenolide-adapted insects are concentrated in the first extracellular loop but may also involve residues of other transmembrane domains. *Left*: Full view of a 3D structure of the pig Na,K-ATPase with bound ouabain (in red and green; Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank 3N23). *Upper Center*: Enlargement of the ouabain binding pocket, indicating the position of amino acid residues implied to be directly involved in ouabain binding (10, 15). *Lower Right*: These residues are compared between the pig and *D. melanogaster*, as representative for all insects of Fig. 1 that are not adapted to cardenolides and compared with insects that typically encounter cardenolides (in blue, relevant substitutions shaded in gray). *Upper Right*: 2D drawing of the ouabain molecule positioned as in the enzyme crystal structure, with the lactone ring deeply buried within the pocket and the sugar moiety facing the surface. Amino acid numbering corresponds to the mature pig enzyme; M1-M2 etc. indicate transmembrane domains and the intermittent extracellular loops of the alpha subunit; β- and γ-subunit of the Na,K-ATPase indicated in the crystal structure cross the membrane once (βM and γM), both are not involved in ouabain binding. A, actutator domain; N, nucleotide binding domain; P, phosphorylation domain; I, isoleucine; G, glycine; F, phenylalanine. Other amino acids and GenBank accession nos. as in Fig. 1.

Mutagenesis studies of the vertebrate enzyme (14, 16) and a recent crystal structure of the pig Na,K-ATPase in its high-affinity ouabain-binding state (11) identified the complete ouabain binding pocket. In addition to Q^{111} and N^{122} it comprises E^{312}, V^{314}, I^{315}, G^{319}, V^{322}, and A^{323} of the M3-M4-hairpin and F^{783}, F^{786}, and T^{797} of the M5-M6 hairpin (Fig. 2). Screening these positions in all of the insects not typically encountering cardenolides (Fig. 1) did not reveal a single difference among them. However, at position 312, all insects consistently differed from the mammalian Na,K-ATPase, by bearing an aspartic acid instead of a glutamic acid. The 17 insects adapted to cardenolides for which the full sequence could be obtained (i.e., all but *C. tenera*) revealed a higher degree of conservation in the C-terminal part of the ouabain binding pocket (Fig. 2). Especially the M3-M4-hairpin is highly conserved and only showed conservative exchanges: the few substitutions observed at position 315 are all among small aliphatic amino acids (i.e., isoleucine, valine, or leucine). In M5-M6, F^{783} is conserved in all species, whereas F^{786} has been substituted for asparagine in the two lygaeid bugs, *L. kalmii* and *O. fasciatus*. The former species also bears a T797A replacement, thus yielding a total of three potentially important substitutions in both species.

The effect of the substitutions Q111L, Q111T, Q111V, and N122H and the combination of Q111T and N122H were tested experimentally by introducing the respective mutations into the Na,K-ATPase α-gene of *D. melanogaster*. Transfecting these constructs into HeLa cells that endogenously express a sensitive Na,K-ATPase and challenging the cells over 48 h with ouabain determines which constructs result in increased ouabain resistance. At 0.5 μM ouabain selection, the double mutant Q111T + N122H resulted in dramatically increased resistance to ouabain. The Q111V replacement and N122H alone also yielded significantly better survival than transfection with the other plasmids. Q111T and Q111L did not increase cell survival compared with transfection with the wild-type *D. melanogaster* Na,K-ATPase gene, and only the former differed significantly from mock transfection with the empty vector (Fig. 3; ANOVA, $F_{6,20}$ = 84.16, $P < 0.001$; Tukey tests at $P < 0.05$). At 0.1 μM ouabain, survival of all cells transfected with the wild-type or mutated *Drosophila* gene was significantly better than for those with the empty vector after 48 h, owing to the increase in Na,K-ATPase expression. Nonetheless, the double mutant again showed demonstrably higher cell survival than all other mutations (Fig. S1). These in turn did not differ significantly at this concentration,

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Table 1. Maximum divergence in pairwise comparisons of Na/K-ATPase sequences among taxonomic groups included in this study

Comparison	Amino acid p-distance	Nucleotide p-distance	Nucleotide synonymous substitutions	Nucleotide nonsynonymous substitutions
Pig–insects	0.221	0.344	0.942	0.165
Among insects	0.127	0.282	0.965	0.097
Among beetles	0.053	0.241	0.856	0.049
Among Lepidoptera	0.030	0.194	0.742	0.022

Columns 2 and 3 give the maximal proportion of different sites (p-distance) for amino acids and nucleotides, respectively; columns 4 and 5 give the maximal proportion of synonymous and nonsynonymous substitutions.

although N122H and Q111V proved to be significantly better than the wild-type *D. melanogaster* gene (Fig. S1; ANOVA, $F_{6,20} = 66.66$, $P < 0.001$, Tukey tests at $P < 0.05$).

Discussion

Striking examples of similar phenotypes caused by identical genetic changes in divergent species are still rare, and for even fewer have functional links been made between the observed changes at the genetic level with phenotypic differences (1, 2). In several instances, molecular similarities go along with adaptations observed at the phenotypic level [e.g., lysozmyes of foregut fermenters (30), prestin genes of echolocating bats and toothed whales (31, 32), and visual pigments of primates and cephalopods (33)]. However, in only few instances was the observed convergence at the genetic level experimentally connected to the functional phenotypes [e.g., for amino acid substitutions in the melanocortin-1 receptor responsible for light coloration in beach mice (34) and in tetrodotoxin-resistant sodium channels in snakes (1, 35)].

For herbivorous insects, our work from natural systems demonstrates a strong pattern and function of molecular substitutions involved in convergent adaptation to host plant defenses. In particular, the deep phylogenetic divergences studied here, across four orders of insects (Fig. 1), demonstrate a profound level of predictability in molecular adaptation across insects that span very diverse feeding guilds. These results provide an important contrast to that from the insecticide resistance literature. For example, resistance to dithiothreitol (DDT) evolved rapidly in response to intense artificial selection, yet was caused by various pathways (36). In contrast, resistance to cyclodiene is based on target site insensitivity by the identical amino acid replacement in at least six insect species on five continents, and it almost certainly evolved repeatedly in some of these species (37). The extent of convergence in molecular evolution thus does not depend on the type of selection or the available time frame. Rather, the likely soft selection caused by prolonged coevolutionary interaction between cardenolide-containing plants and herbivores has resulted in similar molecular convergence as the intense selection by some, but not all, pesticides.

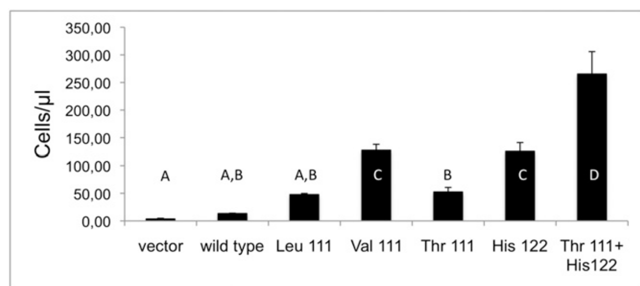

Fig. 3. Survival of HeLa cells (mean of three replicates ± SD) transfected with plasmids bearing the *D. melanogaster* Na,K-ATPase α-subunit either as wild-type or with different amino acid substitutions as indicated. Cells were selected over 48 h with 0.5-µM ouabain added to the medium. Bars marked with different letters differ significantly at $P < 0.05$ (Tukey tests).

Insects specializing on cardenolide-containing plants as hosts have evolved various adaptations to circumvent the toxic effect of these compounds. Indeed, because many such plants, especially in the Apocynaceae, produce copious latex (with concentrated cardenolides), several chewing herbivores have convergently evolved behavioral mechanisms to sever the canals that deliver the latex (38). Target-site insensitivity of the Na,K-ATPase is another counter-strategy. Although substitutions in the mammalian enzyme affecting sensitivity to cardenolides have received continued attention (reviewed in ref. 28), much less is known about structure–function relationships for the insect Na,K-ATPase. This lack of knowledge is perhaps surprising, given that likely hundreds of species of insect herbivores have specialized on cardenolide-containing plants.

Specific Molecular Substitutions in Insect Na,K-ATPase. No other substitutions at position 122 than the exchange of histidine for asparagine have ever been reported for insects, and yet this replacement must have evolved at least four times independently in butterflies, beetles, bugs, and flies. Nonetheless, increased ouabain resistance by an N122H replacement has never been reported in the extensive mutagenesis studies of the vertebrate enzyme (12). In the rat Na,K-ATPase, resistance to ouabain is achieved by a substitution of N122D that occurs in conjunction with an exchange Q111R, resulting in two polar residues being replaced by charged residues (12, 14). Our screen revealed four instances in which the substitution N122H is accompanied by replacements at position 111: three times by Q111V, and once by Q111T in *O. fasciatus* (Fig. 2). Similar to the situation in mammals, the combination of substitutions at both positions, Q111T plus N122H as observed in *O. fasciatus*, conferred the greatest resistance to ouabain in our cell survival assay, and it can safely be assumed that the same also applies to the combination of Q111V plus N122H. However, concurrent substitutions at position 111 were not detected in our sequences of *L. kalmii* and *L. asclepiadis*, which both bear the N122H exchange. Even in isolation this substitution significantly increased cell survival in our assay, although not as effectively as in combination with Q111T.

At position 111, we observed a total of four distinct substitutions in isolation: valine, leucine, threonine, or glutamic acid were replaced in nine species for the conserved glutamine without a concurrent N122H mutation. Our cell survival assay supports that at least the Q111V mutation may increase resistance to cardenolides to a similar extent as the isolated N122H mutation. The substitutions Q111L and Q111T resulted in greater than threefold better cell survival than the wild-type *D. melanogaster* gene, but these differences were not significant. The relative ineffectiveness of the Q111L substitution observed here agrees with a recent in vitro study on Na,K-ATPase susceptibility to inhibition by ouabain (39). This study showed that the enzymes of three arctiid moths investigated here are all highly sensitive to cardenolides, with an IC_{50} of roughly 5 µM ouabain, no matter whether they bear a Q111L exchange

(*Pygoctenucha terminalis*) or have the conserved Q^{111} (*Cycnia oregonensis* and *Euchaetes egle*). The leucine observed at position 111 in several insects not typically encountering cardenolides (Fig. 1) and some species of Danainae (40) thus apparently does not bear on cardenolide sensitivity.

For the remainder of the positions in the first extracellular loop that have been described as being connected to cardenolide sensitivity in vertebrates (12, 29), only position 118 bore substitutions among our insects: in *C. auratus* a P118A replacement was observed, and in *O. fasciatus* P118S. Like the substitution of Q111E observed in the *Largus* species, the effects of these exchanges still remain unclear.

On the basis of the assumption that the amino acids that have been pinpointed as forming the ouabain binding pocket in the mammalian Na,K-ATPase (11, 16) have the same function in insects, we also screened the C-terminal part of the binding pocket (Fig. 2). This assumption needs further investigation but is supported by the high degree of sequence conservation that can be observed in these parts of the enzyme. Some substitutions observed in the C-terminal part of the presumed binding pocket are among similar amino acids and may possibly not change its binding characteristics (e.g., the consistent replacement of E312D in all insect sequences or the substitutions of I315V and I315L in some of the cardenolide-adapted species). In contrast, the substitution of T797A observed in *L. kalmii* has been described as one of the most potent exchanges in the mammalian enzyme providing greatest resistance to ouabain (12, 41, 42) and is expected to have a similar effect here.

Coping with a Potent Plant Toxin. Although our study shows that several insects adapted to cardenolides may have evolved effective target site insensitivity by a limited set of substitutions in their Na,K-ATPase α-subunits, some of the species tested here, the as yet undetermined Mexican flea beetle, and the arctiid moths *E. egle* and *C. oregonensis*, do not show any conspicuous substitutions. As discussed above, we know for the latter two species that they actually possess cardenolide-sensitive Na,K-ATPase (39). Alternatively, toxic effects of ingested cardenolides may be avoided by other mechanisms than target site insensitivity (43). Uptake of cardenolides into the body cavity may be restricted by impermeable guts or highly efficient excretion, as earlier studies have shown (44) and as tracer uptake studies in the non–cardenolide-adapted *C. asclepiadeus* indicate (45). Highly efficient excretion mechanisms have been demonstrated for the Malphigian tubules of *D. melanogaster* (46). Finally, compartmentalization of cardenolides in the body may help protect sensitive tissues like the nervous system against toxic effects (39, 47). These mechanisms are not mutually exclusive but may also occur in combination.

In summary, we have reported a high level of molecular convergence in specific genetic substitutions of the α-subunit of Na,K-ATPase among four orders of insect herbivores adapted to cardenolide-containing plants. These adaptive changes in a gene of major effect are causally linked to reduced sensitivity to cardenolides. Although the costs of this target site insensitivity and its complementarity to other physiological adaptations to cardenolides are unknown, it is clearly a highly repeatable path in molecular adaptation across a wide swath of insects. However, the identity and number of substitutions vary among species, and it is thus far unclear whether this range of substitutions, in an otherwise highly conserved gene, corresponds to reduced sensitivity to the concentration or specific types of cardenolides.

Materials and Methods

Study Organisms. Eighteen insect species of the North American milkweed fauna were collected from cardenolide-containing *Asclepias* and *Apocynum* species (Apocynaceae). In addition, we included three related insect species that can be assumed to be unadapted to cardenolides, because their usual host plants do not contain any (Fig. 1 and Table S1). All insects were shipped to Hamburg in EtOH, RNAlater, or in few exceptions alive and stored at −80 °C until extraction. DNA and RNA were extracted following the protocols of the respective Qiagen tissue kit. The identity of larvae of *L. asclepiadis* (Diptera, Agromyzidae) collected from *Asclepias syriaca* near Ithaca, NY, and a pupa of *Liriomyza* sp. collected from *Asclepias angustifolia*, near Arizpe, Sonora, Mexico, were verified by sequencing 530 bp of their cytochrome c oxidase subunit I (COI) genes (EMBL-Bank accession nos. HE862403 and HE862404). The sequences differed by 33.1% (p-distance) at their third and 2.3% at their first and second positions, corroborating that the individuals belong to distinct *Liriomyza* species [compare data on Agromyzidae (48)].

Sequencing of Na,K-ATPase Genes. To amplify the parts of the Na,K-ATPase that have been described to be involved in ouabain binding in mammals, a set of primers covering amino acid residue 66–813 (Table S2; numbering of amino acids based on the pig enzyme) were used in different combinations with varying DNA concentrations and PCR protocols. Typically the most successful approach was to amplify first the whole region with primers S409 and rATPc and to reamplify smaller fragments for sequencing. Because the gene sequences of the examined insect orders have differing length and positions of introns, we quickly abandoned the DNA-based approach used by previous workers (19, 23–26) and used RT-PCR instead. RNA was transcribed into cDNA with a 17polyT-primer and SuperScript III reverse transcriptase (Invitrogen) according to manufacturer protocols. PCR products were cleaned directly or after excision from agarose gels (PCR purification kit; Qiagen), cycle sequenced (BigDye-Terminator Kit; Applied Biosystems), cleaned from remaining enzyme and nucleotides (DyeEX columns; Qiagen), and electrophoresed on an automated sequencer (ABI Prism 3100 Genetic Analyzer; Applied Biosystems). Sequence fragments were assembled with Sequencher 4.6 (Gene Codes) and compared with sequences deposited in GenBank using the BLAST algorithms. Additional sequences of the homologous Na,K-ATPase α-subunit gene of various insects were retrieved from GenBank. A final alignment and analysis of nucleotide and corresponding amino acid sequences was achieved with Mega 5.05 (49) and analyzed for their amino acid content and general divergence parameters.

In Vitro Expression of Mutants. To test the effect of individual substitutions in the Na,K-ATPase, we cloned the coding sequence of the α-subunit of the Na,K-ATPase of wild-caught *D. melanogaster* from Hamburg, Germany. Total RNA was extracted and transcribed into cDNA as described above. The coding sequence of the Na,K-ATPase α-subunit was amplified with the primers 5′-GAGAATGCCGGCCAAAGTTAATAAAAAGG-3′ and 5′-GAGATTAATAGTAGGTCTCCTGCTCCAACC-3′ using a proofreading Taq (Platinum Pfx DNA Polymerase; Invitrogen). The PCR product was ligated into a TA cloning vector (Topo TA cloning kit; Invitrogen), control sequenced, excised with BamHI and XhoI (Fermentas), and cloned into a pcDNA3.1$^+$ expression vector (Invitrogen). The gene sequence corresponds to GenBank accession no. AF044974 (27), with the exception of four silent substitutions and a substitution of a valine for an alanine codon at position 1759 of AF044974 (corresponding to amino acid 522 of the pig enzyme) and is deposited under EMBL-Bank accession no. HE962487. The amino acid codons of interest were introduced into this construct by site-directed mutagenesis (Quick Change II Kit; Stratagene) according to the manufacturer protocol. All constructs were sequenced to control for correctly introduced mutations. The mutated and wild-type Na,K-ATPase plasmids, as well as the empty vector, were transfected with X-tremeGENE transfection reagent (Roche Applied Science) into HeLa cells.

Survival assays (14, 19) were carried out in 24-well plates starting with 0.7×10^5 cells per well. After 24 h three wells on each of three plates were transfected with the same plasmid. Twenty-four hours after transfection the number of living cells was determined for one of the plates by propidium iodide staining and flow cytometry (Guava easyCyte; Merck Millipore). Ouabain (a commercially available, polar cardenolide; Sigma) (0.1 μM or 0.5 μM) was added to the medium of all wells of one plate each, and the number of surviving cells was determined again after 48 h of ouabain selection. HeLa cells endogenously express high levels of Na,K-ATPase, and those with only ouabain-sensitive forms will die within a few days. Each assay consisted of three replicates for both concentrations and each tested plasmid. Differences in the survivorship after 48 h of ouabain treatment of cells transfected with different plasmids were evaluated by ANOVA.

ACKNOWLEDGMENTS. We thank Helga Pankoke, Deane Bowers, and Georg Petschenka for providing insects; G. Petschenka and Deane Bowers for the identification of *C. oregonensis* and *P. terminalis*; Peter Iglauer and Ingo Narberhaus for help with construction of the expression plasmid; and Rick Harrison, Georg Petschenka, and anonymous reviewers for comments on the manuscript. This study was supported by Grant Do527/5-1 (to S.D.).

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