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Inhibition of Na^+,K^+-ATPase by the Cardenolide 6′-*O*-(*E*-4-Hydroxycinnamoyl) Desglucouzarin

Alan J. Abbott, Charles G. Holoubek, and Ronald A. Martin
Department of Chemistry & Physics, Louisiana State University at Shreveport, Shreveport, Louisiana 71115

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Among the major cardenolides from the milkweed *Asclepias asperula*, 6′-*O*-(*E*-4-hydroxycinnamoyl) desglucouzarin has not been characterized biochemically. In this study, its binding affinity for a physiological receptor, porcine kidney Na^+,K^+-ATPase, was found to be lower than the other cardenolides in this plant. The order of affinities from highest to lowest was: uzarigenin (K_d = 1.05 μM) = desglucouzarin (K_d = 0.98 μM) > uzarin (K_d = 4.0 μM) > 6′-*O*-(*E*-4-hydroxycinnamoyl) desglucouzarin (K_d = 16 μM). The chemical attachment of the 4-hydroxycinnamoyl group to the 6′-carbon of desglucouzarin significantly inhibits binding. This agrees with predictions that a 5′-methyl group on cardenolides fits the receptor site optimally for the porcine kidney enzyme. The 4-hydroxycinnamic ester was also found to be fluorescent.

Cardenolides are a group of C_{23} steroids produced in nature by several plant families and some species of toads. Cardenolides in one of the major plant families, Asclepiadaceae, are well known causes of death in sheep and cattle in open range grazing. Because a major site of action is the heart, cardenolides are also known as cardiac glycosides. Despite their toxicity, some cardiac glycosides have therapeutic effects and, at the appropriate dose, have been used as a treatment for conjestive heart failure (1). In 1991 the cardenolide content from Asclepiadaceae milkweeds from the Northwest region of Louisiana were examined and a unique cardenolide was discovered among 5 that were previously reported (2). The compound was 6′-*O*-(*E*-4-hydroxycinnamoyl) desglucouzarin which will be referred to as HCD. Although the parent structure, desglucouzarin, was a known cardenolide, the functional group, 4-hydroxycinnamic acid was unique among cardenolides. This report examines the effect of HCD on its pharmacological receptor.

The only known receptor for cardenolides is the integral membrane protein Na^+,K^+-ATPase. This protein actively transports Na^+ and K^+ across the plasma membrane of most higher eukaryotic cells using the energy of ATP hydrolysis. It is a glycoprotein composed of a 110 kD α-subunit and 50 kD, glycosylated β-subunit (3). Cardenolides bind the enzyme region exposed at the outer surface of the cell membrane but the molecular details of binding site have not been entirely established (4). One approach to studying the cardenolide binding site has been to search for the common structural features shared by all cardenolides with high affinity for the enzyme. This has been done for many of the hundreds of known cardenolides, both synthetic and natural (5). The minimum structure required is a steroid ring system with 14-β-hydroxyl group and 17-β-unsaturated lactone ring (figure 1). At carbon 3, a glycosidic link to a monosaccharide containing a 5′-methyl group and 4′-hydroxyl usually enhances further binding (6, 7). The unique 4-hydroxycinnamoyl group of HCD effectively modifies the 5′-methyl group that enhances binding. In this report, the HCD affinity for the enzyme was found to be 16 times weaker than desglucouzarin. This data supports the view that a bulky group on carbon 5′ of the sugar group in a cardenolide is very disruptive to binding the porcine kidney enzyme. The 4-hydroxycinnamic ester was also found to be fluorescent.

MATERIALS AND METHODS

Chemicals. Fluorescein-5′-isothiocyanate (FITC) was from Molecular Probes (Eugene, OR). All other buffers and salts and chromatography media were obtained from Sigma Chemicals (St. Louis, MO).

Isolation of cardenolides. All cardenolides were isolated from the milkweed *Asclepias asperula* as described previously (Martin et al., 1991). These include uzarin, desglucouzarin, uzarigenin and 6′-*O*-(*E*-4-hydroxycinnamoyl) desglucouzarin (referred to as HCD). Concentrations of stock cardenolide solutions were determined by a colorometric assay based on the blue complex cardenolides form with TNDP (8).

Isolation of enzyme. The Na^+,K^+-ATPase enzyme was isolated according to the method of Jorgensen (9) from fresh porcine kidneys that were obtained locally. It was stored at 4°C until ready to use. The initial specific activity for Na^+, K^+ dependent ATP hydrolysis was 1100 μmolPi/mg/hr as measured by the coupled enzyme assay described below. On SDS-PAGE gels the enzyme displayed predom-

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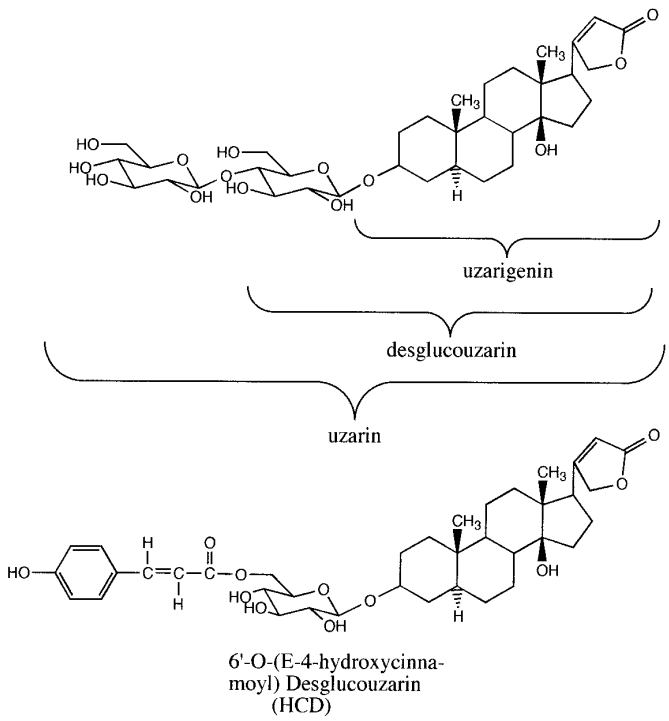

FIG. 1. Structural formulas for an uzarigenin based cardenolide series found in *Asclepias asperula* (2). Uzarigenin and 3 natural derivatives isolated from the milkweed *Asclepias asperula* (2) and tested in this study are shown. The heretofore uncharacterized cardenolide activity of 6′-*O*-(*E*-4-hydroxycinnamoyl) desglucouzarin (HCD) is found to be the weakest of the series based on inhibition of the enzyme Na^+,K^+-ATPase.

inantly 2 bands correlating with the molecular weight of the enzymes α-subunit (110 kD) and β-subunit (55 kD). The Bradford assay (10) was used to determine protein concentration.

FITC labeling of enzyme. The procedure is essentially the same used previously (11). Ten micromolar FITC was added to 2 mg/ml enzyme in Trizma buffer, pH 9.0 and incubated for 30 minutes in the dark at room temperature. Unbound label was removed by gel filtration over a 10 fold volume excess G-100 Sephadex (10 ml column).

Fluorescence measurements. All fluorescent measurements were made with a Perkin-Elmer (Foster City, CA) model LS-5B steady-state fluorescence spectrometer. Fluorescence spectra of HCD were obtained with 1 micromolar HCD in 0.05 M Trizma Buffer, pH 7.5. Excitation spectra recorded 431 nm emission while excitation wavelengths were scanned 250-400 nm. Emission spectra were recorded over 350-550 nm while exciting at 300 nm. Both spectra were normalized to a maximum value of 1.0. The fluorescence of the FITC labeled enzyme was measured for 10-50 ug of labeled enzyme in 1.0 ml Trizma buffer, pH = 7.5 containing 2 mM $MgCl_2$. All measurements were at room temperature with a 480 nm excitation wavelength and 520 nm emission wavelength.

Enzyme inhibition assay. Enzyme activity was measured using the coupled assay described previously (12). The oxidation of NADH is coupled to the enzyme catalyzed hydrolysis of ATP. The velocity of [NADH] decrease is measured by absorbence at 340 nm in units of A/s. This was converted to μmol Pi/hr using the extinction coefficient 6220 M^{-1} s^{-1} for NADH, 1050 μl as volume and converting s^{-1} to hr^{-1}. All assays were performed at 37°C. For specific activities, the velocity in μmol Pi/hr was divided by the enzyme weight in milligrams (see enzyme isolation). Inhibition was measured as the relative loss in enzyme activity upon addition of cardenolide:

$$\% \text{ inhibition} = \frac{(V_0 - V_i)}{V_0} \times 100\%,$$

where V_0 and V_i are activities (velocities) before and after addition of cardenolide, respectively. Percent inhibition data at several cardenolide concentrations was fit to the following single site binding equation:

$$\% \text{ inhibition} = \frac{[D]}{K_d + [D]} \times 100\%$$

Curve fitting was accomplished using the graphing program, Kaleidagraph, by Synergy Software (Reading, PA). This model for binding has been reported for other cardenolides (13).

RESULTS

A. Spectroscopic Properties of 6′-O-(E-4-Hydroxycinnamoyl) Desglucouzarin

Absorbance. The hydroxycinnamoyl moiety absorbed at 316 nm wavelength with a previously reported extinction coefficient of 29,512 $M^{-1}cm^{-1}$ (2). Its reaction with 2,2′,4,4′-tetranitrodiphenyl (TNDP) in a basic ethanol solution produced absorbance at 626 nm that is characteristic of cardenolides carrying an unsaturated lactone ring (8). Both properties could be used to reliably determine the cardenolide concentration (see Materials and Methods).

Fluorescence. The hydroxycinnamoyl moiety also conferred significant fluorescence to the cardenolide. Figure 2 shows excitation maxima of 322 nm and emission maxima at 430 nm. The occurrence of fluorescence makes this moiety a potential probe of the physical and chemical environment of the cardenolide binding site. Unfortunately, we would need 16 μM enzyme to bind 1/2 of all HCD on solution (i.e. K_d = 16 μM). We added 100 nM enzyme to 10 μM HCD and saw no fluorescence

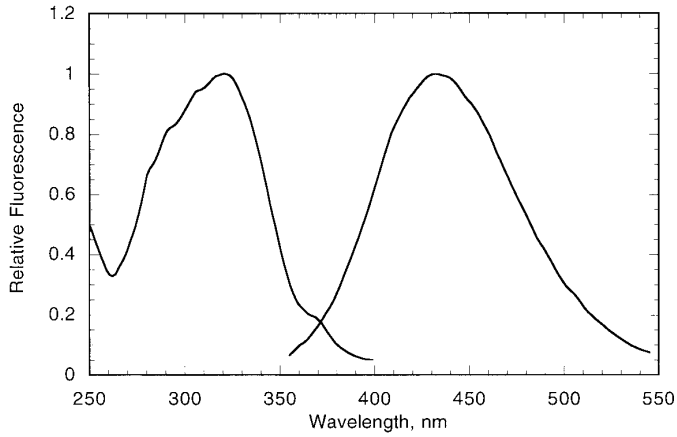

FIG. 2. Fluorescence properties of 6′-*O*-(*E*-4-hydroxycinnamoyl) desglucouzarin (HCD). Uncorrected fluorescence excitation and emission spectra show excitation λmax = 322 nm and emission λmax = 431 nm.

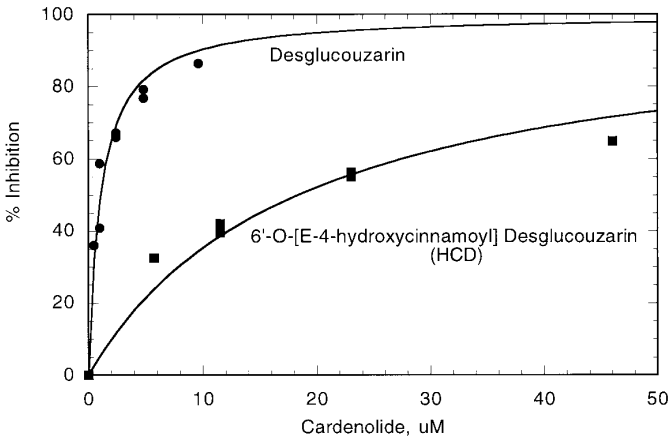

FIG. 3. Effect of 4-hydroxycinnamoyl group on the binding of desglucouzarin. The concentration dependence for the binding of desglucouzarin to the enzyme Na^+,K^+-ATPase is compared to the same compound with a 4-hydroxycinnamoyl group (see HCD, figure 3). Best fit lines are for the single site binding equation, $\%I = 100 \times [\text{cardenolide}]/(K_d + [\text{cardenolide}])$, where $\%I$ is the percent of inhibition of enzyme activity. Binding constants are 0.94 μM ($\pm$ 0.08 μM) for desglucouzarin and 16 μM ($\pm$ 1.5 μM) for HCD. HCD concentrations were limited by solubility to about 60 μM.

change. Assuming that a maximum of 1% of all HCD could have been bound we can only say that a large ($\geq$10 fold) increase in quantum yield was not observed.

B. Effect of 6′-O-(E-4-Hydroxycinnamoyl) Desglucouzarin on Na^+,K^+-ATPase

HCD altered enzyme structure and activity in a manner that is characteristic of cardiac glycosides (3). Accordingly, the Na^+, K^+ dependent ATPase activity was inhibited by HCD with a concentration dependence that was well described by a single site binding equation (figure 3). Enzyme labeled with fluorescein at or near its ATP binding site shows a magnesium dependent loss in fluorescence when cardiac glycosides bind. This is also observed for HCD and all cardenolides used in this study (data not shown). These observations along with its original structural elucidation (2) led us to believe that HCD was indeed a cardiac glycoside that binds to Na^+,K^+-ATPase at its known binding site.

C. Contribution of 4-Hydroxycinnamoyl Moiety of HCD to Cardiac Glycoside Activity

The unique chemical structure on HCD is its 4-hydroxycinnamoyl moiety. To assess this group's contribution to enzyme inhibition, the binding isotherm for HCD was compared to that of its parent compound, desglucouzarin (figure 3). Figure 1 shows the relevant chemical structures. The enzyme had considerably lower affinity for HCD compared to desglucouzarin, based on the 16 fold higher concentration required for half-maximal inhibition. We conclude that the 4-hydroxy cinnamoyl moiety at this position (6′ carbon) had a negative effect on enzyme binding.

HCD binding was also compared within a structural series of cardenolides based on the parent aglycone uzarigenin. It included uzarigenin and its glycosides, desglucouzarin (3-O-glucose side chain), HCD (3-O-glucose-6′-*O*-[*E*-4-hydroxycinnamoyl] side chain) and uzarin (3-O-glucose-4′-O-glucose side chain). All binding data were fit to a single site equilibrium binding model. Table 1 shows that K_d stayed about the same or increased with degree of glycosylation (uzarigenin = desglucouzarin < uzarin). This agrees well with previous studies using these compounds (14, 5). HCD showed the highest K_d, and thus, bound the poorest of all cardiac glycosides in this series. The 4-hydroxycinnamoyl derivative on C6′ of glucose in desglucouzarin is more disruptive of binding than the derivative with an extra glucose attached to the C4′ position of desglucouzarin.

DISCUSSION

Cardenolides found in nature are natural toxins and emetics that protect plants and insects from predation. In humans and other mammals they have cardiotonic activity which is the basis for much of their pharmacological uses. These compounds have in common a steroid ring system linked to an unsaturated lactone ring at C17 and 1-3 sugar residues O-linked to carbon 3 of the steroid (1). Among the hundreds of known cardenolides, only 6′-*O*-(*E*-4-hydroxycinnamoyl) desglucouzarin (HCD) carries the 4-hydroxycinnamoyl functional group (2). This is the first report its biological activity. We have found that HCD binds Na^+,K^+-ATPase, inhibits its activity and alters its structure in a way that is characteristic of cardiac glycosides. In addition, the 4-hydroxycinnamoyl functional group in HCD increases the K_d for binding 16 fold compared to its parent compound, desglucouzarin. We have also found that this cardenolide is fluorescent.

TABLE 1

Equilibrium Dissociation Constants
Uzarigenin-Based Cardenolides

Compound[1]	K_d (μM)[2]
uzarigenin	1.05 $\pm$ 0.04
desglucouzarin	0.94 $\pm$ 0.08
HCD[3]	16 $\pm$ 1.5
uzarin	4.0 $\pm$ 0.40

[1] Compound structures are listed in Figure 1.
[2] Data analogous to Figure 3 was fit to the equation, $\%I = 100 \times [\text{cardenolide}]/(K_d \times [\text{cardenolide}])$.
[3] HCD is 6′-*O*-(*E*-4-hydroxycinnamoyl) desglucouzarin.

The effect of the 4-hydroxycinnamoyl group on cardenolide activity. Cardenolide activity was measured by the affinity of cardenolides for their physiological receptor, Na^+,K^+-ATPase. The affinity of the parent compound, desglucouzarin, was in reasonable agreement with the value reported for the guinea pig heart enzyme. The change in K_d when a glucose moiety was added to desglucouzarin (to form uzarin) or removed from desglucouzarin (to form uzarigenin) also agreed with this report (15). The 16 fold increase in K_d induced by the 4-hydroxycinnamoyl group esterified to desglucouzarin (see figure 3) is the largest effect seen for any functional groups in this study.

Implications for structure-activity relationships. Reviewing the effects of many carbohydrate structures on cardenolide activities, Thomas et al. (5) found that 5′ deoxy sugars, those with a 5′-methyl group instead of the usual 5′-hydroxymethyl group, caused the greatest increase in affinities compared to their aglycones. Other small group modifications of sugar moieties at their 2′,3′ and 4′ carbons generally decreased binding affinity . This is true of both the uzarigenin type aglycone and the digitoxigenin type aglycone which differ by the stereo configuration at the A/B ring junction of the steroid ring system (5, 14, 15). Presumably, these 5′ deoxy sugars optimally match the contours of the binding site and contribute to specific hydrogen bond interactions via hydroxyl groups on 2′,3′ and 4′ carbons.

Adding relatively large chemical groups to monosaccharide units, apparently, extends the immediate monosaccharide binding site to include other positive or negative interactions. For example, linking an anthroyl group (15 carbons) to the 3′ carbon slightly increased the affinity of the cardenolide ouabain (16) while acetylation of the same carbon in other cardenolides had the opposite affect (6). Similarly, glycosylation (6 carbons, 5 oxygens) at the 4′ carbon increases affinity in some instances whereas acetylation decreases it (5). Our results suggest that the large 4-hydroxycinnamoyl group esterified to the 5′ hydroxymethyl of glucose causes a significant decrease in affinity.

Comparing our results to similar cardenolides with other 5′ substituents we find the affinities increasing in the following order $-CH_2$-4-hydroxycinnamoyl $<$ $-CH_2OH$ $<-CH_3$. These results are consistent with the hypothesis that the sugar binding subsite is sterically matched to a 5′ methyl group but too small to accommodate the larger groups (5, 14). Interestingly, the enzyme from some species show a different effect, they increase affinity for cardenolides when the 5′ methyl group is a hydroxy methyl group (14). They observed this for the rat and cat enzymes which have generally low cardenolide affinity but not for the high affinity porcine kidney enzyme used in this report. Presumably, when the sugar subsite on the enzyme includes regions in contact with the 4-hydroxycinnamoyl group, no unique favorable interactions are gained.

The fluorescence of HCD was readily detectable but could not be used here for structural studies. Unreasonably high enzyme concentrations, 16 μM, would be required to bind even half the cardenolide in solution. From our 100 nM enzyme observations we estimate that the bound cardenolide has a $\leq$10-fold increase in quantum yield upon binding. Fluorescent properties of this group may prove useful if it is synthetically attached to a higher affinity cardenolide.

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