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COMPARATIVE ABILITY OF DIGOXIN AND AN AMINOSUGAR CARDIAC GLYCOSIDE TO BIND TO AND INHIBIT Na^+,K^+-ADENOSINE TRIPHOSPHATASE

EFFECT OF POTASSIUM*

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Abstract—We compared the abilities of digoxin and aminogalactose digitoxigenin (ASI-222) to bind to, or inhibit, purified dog heart Na^+,K^+-ATPase in the presence of 1, 10, or 80 mM potassium chloride. Changing the potassium concentration from 1 to 10 mM increased the dose producing 50% inhibition of enzyme activity (IC_{50}) by 9- and 2.5-fold for digoxin and ASI-222 respectively. Raising the potassium concentration to 80 mM increased the IC_{50} for digoxin 3-fold but did not alter significantly the IC_{50} for ASI-222. Equilibrium binding studies showed that this enzyme exhibited a single class of specific binding sites for both digoxin and ASI-222. Raising the potassium concentration did not affect the maximum number of binding sites (B_{max}) but increased the apparent dissociation constant (K_D) for digoxin. Potassium differentially affected the affinity and number of binding sites for ASI-222; raising the potassium concentration from 1 to 10 mM did not affect the B_{max} or the K_D, but raising it to 80 mM increased both. The effect of i.v. infusion of potassium chloride upon cardiac arrhythmias produced by i.v. infusion of digoxin or ASI-222 in anesthetized dogs was also determined. Infusion of potassium chloride reversed the cardiac arrhythmias due to digoxin to normal rhythm, but not those due to ASI-222. In conclusion, the interaction of digoxin and the polar digitalis agent, ASI-222, with dog heart Na^+,K^+-ATPase was differentially affected by potassium. These agents also produced cardiac arrhythmias, which were differentially affected by potassium.

It is widely accepted that cardiac glycosides exert their inotropic actions by binding to and inhibiting sodium, potassium adenosine triphosphatase (Na^+,K^+-ATPase§) [1–3]; direct cardiac toxicity also appears to result from inhibition of the enzyme [1]. Numerous reports have described the binding characteristics of cardiac glycosides to Na^+,K^+-ATPase obtained from both digitalis-sensitive and -resistant species [4–6]. Single and, recently, multiple classes of specific binding sites have been described for various digitalis agents in different species [4–10]. Potassium stimulates Na^+,K^+-ATPase activity and also reduces binding of cardiac glycosides to Na^+,K^+-ATPase [11–13]. Chronic exposure to potassium *in vitro* increases the number of specific ouabain binding sites in cultured chick heart cells [14]. However, chronic dietary intake of excessive potassium decreases Na^+-pump site numbers in rat tail arteries [15].

We investigated the cardiac and biochemical actions of a polar, semisynthetic, aminosugar derivative of digitalis, ASI-222. This agent has about a 10-fold greater potency than neutral-sugar digitalis in inhibiting Na^+,K^+-ATPase of pig brain and the Na^+-pump of isolated dog heart myocytes. ASI-222 also demonstrates a greater potency in increasing contractile force and movement in dog whole heart and isolated myocytes [5, 16, 17]. Aminosugar digitalis agents produce a profile of cardiac toxicity that lacks the component of sympathetic nervous stimulation noted for neutral sugar digitalis agents [17, 18]. In conscious dogs, we determined that the increase in cardiac contractility to ASI-222 is more prompt and of shorter duration than to digoxin; comparable levels of contractility to the cardenolides are maintained with about 10-fold lower plasma levels of ASI-222 than digoxin [19]. Cardiac arrhythmias from toxic doses of digitalis agents involve inhibition of the cardiac Na^+,K^+-ATPase [20]. These arrhythmias can be reversed by administering potassium chloride [21, 22].

We compared the characteristics of [^{3}H]digoxin and [^{3}H]ASI-222 in binding to and inhibiting a partially purified dog heart Na^+,K^+-ATPase preparation, and we investigated the effect of potassium chloride on these processes. In anesthetized dogs, we also compared the effect of potassium chloride on patterns of cardiac toxicity to digoxin and ASI-222 given by i.v. infusion. Our results indicate that

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§ Abbreviations: Na^+,K^+-ATPase, sodium, potassium adenosine triphosphatase; K_D, dissociation constant; B_{max}, maximum binding; IC_{50}, median inhibitory concentration; and TCA, trichloroacetic acid.

digoxin and ASI-222 differ in their ability to bind to and inhibit dog heart Na^+,K^+-ATPase. Altering potassium in the incubation medium had different results on enzyme inhibition by, and binding properties of, these two digitalis agents. Furthermore, potassium reversed the cardiac toxicity produced by digoxin but not by ASI-222.

METHODS

Enzyme studies. The partially purified Na^+,K^+-ATPase preparation used in these experiments was obtained from dog heart as described by Pitts *et al.* [23]. In summary, a microsomal pellet, prepared from approximately 100 g of ventricular muscle, was subsequently treated with sodium iodide, sodium deoxycholate, and glycerol to enhance the specific activity. The final pellet was dialyzed overnight at 4° against imidazole: EDTA buffer (25 mM: 1 mM); aliquots of this preparation were stored at −70° until use. The specific activity of this enzyme preparation was 40–70 μmol P_i released/mg protein/hr. About 96% of this enzyme activity was inhibited by 1 mM ouabain. Protein concentration was determined by the method of Lowry *et al.* [24] using bovine serum albumin as standard.

The enzyme activity was determined by incubating 10–15 μg of enzyme protein at 37° in 1 ml of a reaction mixture containing 50 mM Tris buffer (pH 7.4), 5 mM $MgCl_2$, 100 mM NaCl, 10 mM KCl, and 0.5 mM EDTA. Various concentrations of digoxin or ASI-222 were added to this reaction mixture. Following a 10-min preincubation period, the reaction was started by adding ATP (5 mM) and was terminated by adding 1 ml of 10% (w/v) trichloroacetic acid (TCA) at the end of a 30-min incubation. Dose–response (concentration–inhibition) curves were constructed for these glycosides. Potassium concentration (as KCl) was also varied to determine the effect of this ion on inhibition of the enzyme by the two glycosides. We did not alter the Na^+ concentration in the media. The inorganic phosphorous (P_i) liberated by the enzyme was determined spectrophotometrically according to the method of Martin and Doty [25]. The IC_{50} values are expressed as micromolar concentrations and are means ± SE from four different experiments unless otherwise indicated. Means from groups treated with 1, 10, and 80 mM K^+ were compared with ANOVA using BMDP software.

To determine the binding characteristics of [^{3}H]glycoside, 5–10 μg enzyme protein was incubated at 37° with various concentrations of [^{3}H]digoxin or [^{3}H]ASI-222 (≈10,000 dpm/pmol) in a medium containing 100 mM NaCl, 5 mM $MgCl_2$, 5 mM ATP, and 50 mM Tris buffer (pH 7.4). To determine the effect of potassium on the binding of the glycosides to the enzyme, KCl was included in the medium (0, 1, 10, or 80 mM). The binding reaction was started by adding [^{3}H]glycoside and was stopped rapidly by filtering the sample through EH Millipore® filters (0.5 μm pore size) on a vacuum manifold. Since the equilibrium binding was achieved within 10 min and maintained for 90 min for both glycosides, the incubation time chosen was 20 min. The filters were washed with 20 ml (4 × 5 ml) of ice-cold distilled water. The amount of tritiated drug bound to protein was determined by counting the radioactivity on the filter in a scintillation counter. Specific binding was a linear function of protein concentration. Nonspecific binding was determined by incubating the enzyme in the medium above (but without ATP) containing [^{3}H]glycoside and excess unlabeled glycoside (10^{-4} M). All values for [^{3}H]ligand binding were corrected for this nonspecific binding. Nonspecific binding was not affected by different K^+ concentrations. All assays were carried out in triplicate. Binding data were examined by Scatchard analysis. Apparent dissociation constants (K_D) and the maximum number of binding sites (B_{max}) were determined. K_D and B_{max} values are means ± SE from four experiments. Values for 1, 10, and 80 mM K^+ were compared with ANOVA using BMDP software.

Whole-dog studies. For studies of cardiac arrhythmias, eight mongrel dogs were anesthetized with sodium pentobarbital, 30 mg/kg, i.v. A polyethylene catheter was inserted into the abdominal aorta to measure blood pressure. Lead II EKG was monitored to assess cardiac electrical events. Both glycosides, dissolved or diluted in saline, were infused at a rate that produced cardiotoxicity in about 80–100 min at volume rates of 2 ml/min. For ASI-222, this rate was 1 μg/kg/min, and for digoxin, 2 μg/kg/min. When cardiac arrhythmias occurred, either 50% occurrence of ectopic beats within 1 min or evidence of second degree A-V nodal blockade, an i.v. infusion of KCl, 5 mEq/kg/min, was begun and maintained until cardiac rhythm reverted to normal, if ever. Plasma potassium concentration was determined for each dog by flame photometry. Samples were taken at the onset of arrhythmias and at the point of reversal of cardiac toxicity, if it occurred.

Drugs. ASI-222 or 3-β-*O*-(4-amino-4,6-dideoxy-β-D-galactopyranosyl) digitoxigenin was obtained from Ash-Stevens, Inc. (Detroit, MI). Digoxin was obtained from the Sigma Chemical Co. (St. Louis, MO). Both [^{3}H]digoxin and [^{3}H]ASI-222 were obtained from New England Nuclear (Boston, MA).

RESULTS

Effect of [K^+] *on inhibition of dog heart* Na^+,K^+-*ATPase by digoxin and ASI-222.* Figure 1A illustrates dose–response (concentration–inhibition) curves for digoxin with 1, 10, and 80 mM potassium KCl. The concentrations of digoxin needed to produce 50% of the maximum inhibition (IC_{50}) were compared at these three K^+ concentrations. Increasing the K^+ concentration in the incubation medium from 1 to 10 mM shifted the concentration–inhibition curve significantly ($P < 0.05$, ANOVA) to the right by 9-fold. Further increasing the K^+ concentration to 80 mM produced a further shift to the right by 3-fold ($P < 0.05$, ANOVA, compared to 1 mM). These findings are reflected in the IC_{50} values shown in Table 1.

Figure 1B represents data for ASI-222. Increasing the K^+ concentration from 1 to 10 mM shifted the concentration–inhibition curve to the right, but only 2.5-fold. This was, however, a significant change ($P < 0.01$, ANOVA). Unlike observations with

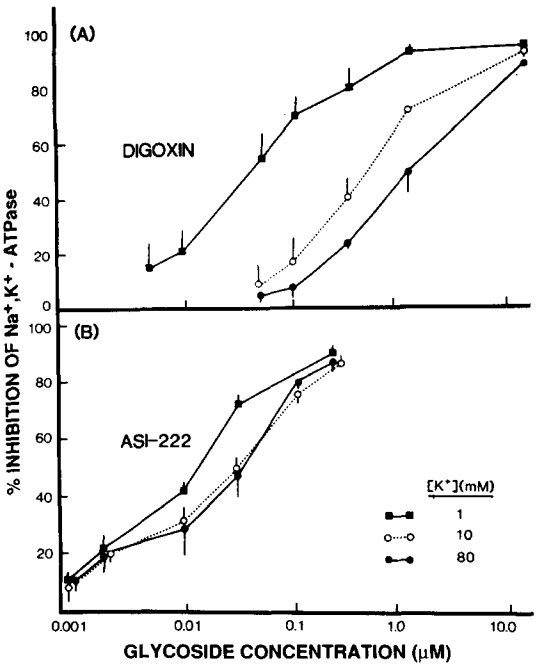

Fig. 1. Percent inhibition of dog heart Na^+,K^+-ATPase in the presence of 1, 10, or 80 mM K^+ by increasing concentrations of digoxin (A) and ASI-222 (B). Data points are means and standard error for six experiments.

digoxin, a further increase in the K^+ concentration to 80 mM did not alter the ability of ASI-222 to inhibit the enzyme. As can be seen from the figure and the IC_{50} values (Table 1), the aminocardenolide ASI-222 was about ten times more potent an inhibitor of dog heart Na^+,K^+-ATPase than digoxin in the presence of 10 mM K^+.

Effect of [K^+] *on binding of* [^{3}H]*digoxin and* [^{3}H]*ASI-222 to dog heart* Na^+,K^+*-ATPase.* An equilibrium in binding of [^{3}H]digoxin and [^{3}H]ASI-222 was reached within 10 min and maintained through 90 min. Therefore, a 20-min period for binding was allowed. Panels A and B of Fig. 2 show specific binding of [^{3}H]digoxin (A) and [^{3}H]ASI-222 (B) to the enzyme in the absence of K^+. Analysis of data indicates that under these conditions both [^{3}H]digoxin and [^{3}H]ASI-222 interact with a single class of binding sites. The maximum number of binding sites, B_{max}, and the dissociation constant, K_D, values for digoxin and ASI-222 binding were 47 and 54 pmol/mg protein and 0.026 and 0.207 μM respectively.

The binding parameters of [^{3}H]digoxin and [^{3}H]ASI-222 to dog heart Na^+,K^+-ATPase in the presence of 1, 10, or 80 mM K^+ are shown in Table 1. These data indicate that an increase in the K^+ concentration from 1 to 80 mM decreased the affinity of binding of [^{3}H]digoxin to this enzyme progressively as reflected in the K_D values, although only the 80 mM K^+ value was significantly different from the 1 mM value ($P < 0.01$). Potassium did not affect the B_{max} (Table 1). Raising the potassium concentration from 1 to 10 mM did not affect the B_{max} or the K_D for ASI-222, but further raising it to 80 mM increased both of these values ($P < 0.001$) (Table 1). The differences between the B_{max} values obtained with no K^+ and those obtained with 1, 10, and 80 mM K^+ (see Table 1 and Fig. 2 legends) were due to the use of two different batches of enzyme with different specific activities in experiments with and without potassium.

Figure 3 is a plot of percent glycoside inhibition of dog heart Na^+,K^+-ATPase as a function of percent glycoside binding to this enzyme in the presence of 10 mM potassium. Data points obtained for digoxin yielded a linear plot with a correlation coefficient of 0.986, suggesting a perfect correlation of inhibition with binding. With ASI-222, however, the enzyme was maximally inhibited at about the 40% binding level. Furthermore, binding continued after enzyme inhibition was completed.

Effect of [K^+] *on cardiotoxicity of digoxin and ASI-222 in anesthetized dogs.* Digoxin produced cardiac

Table 1. Comparison of digoxin and ASI-222 in terms of their binding to and inhibiting dog heart Na^+,K^+-ATPase: effect of potassium

[K^+] (mM)	IC_{50} (μM)	K_D (μM)	B_{max} (pmol/mg)
	Digoxin		
1	0.048 ± 0.012	0.108 ± 0.009	87.6 ± 3.6
10	0.380 ± 0.065*	0.445 ± 0.084	91.9 ± 10.7
80	1.150 ± 0.343*	0.884 ± 0.199†	72.8 ± 8.1
	ASI-222		
1	0.014 ± 0.003 (8)	0.416 ± 0.04	108.3 ± 2.8
10	0.038 ± 0.007† (6)	0.501 ± 0.04‡	116.4 ± 2.3‡
80	0.041 ± 0.006†	1.229 ± 0.08§	177.2 ± 9.2§

Values are means ± SE for four experiments each, except when indicated in parentheses. ANOVA was used to compare the values obtained for 1, 10, and 80 mM potassium.

* $P < 0.05$ compared to 1 mM [K^+].

† $P < 0.01$ compared to 1 mM [K^+].

‡ $P < 0.001$ compared to 80 mM [K^+].

§ $P < 0.001$ compared to 1 mM [K^+].

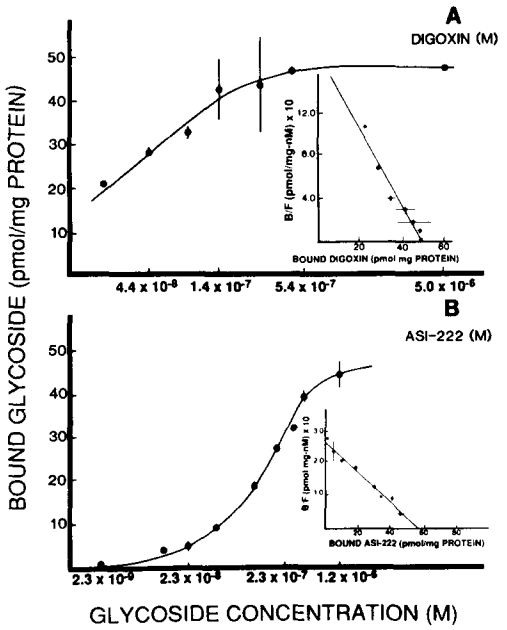

Fig. 2. Specific binding of [^{3}H]digoxin (A) and [^{3}H]ASI-222 (B) to dog heart Na^+,K^+-ATPase in the absence of K^+. The insets represent the Scatchard plots derived from these data. Data points are the means and standard errors of five experiments for each compound. The K_D and the B_{max} values derived from these plots are 0.026 and 0.207 μM and 47 and 54 pmol/mg protein for digoxin and ASI-222 respectively.

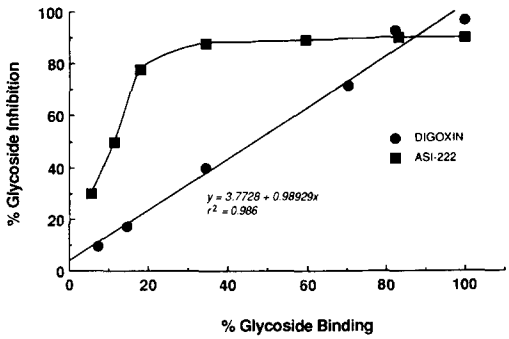

Fig. 3. Glycoside inhibition as a function of glycoside binding. Data points are percent inhibition of dog heart Na^+,K^+-ATPase versus percent binding in the presence of 10 mM potassium.

arrhythmias at approximately 120 μg/kg compared to about a 100 μg/kg dose of ASI-222 required for toxicity. An intravenous infusion of KCl at a dose-rate of 5 mEq/kg/hr reversed the cardiac arrhythmias due to digoxin in all dogs after an average total dose of 0.18 mEq/kg (≈6 min). However, administration of KCl during cardiac arrhythmias due to ASI-222 never resulted in reversion to a normal sinus rhythm (Table 2).

Plasma potassium concentrations at the beginning point of cardiac toxicity were similar for the two groups. At the time at which cardiac rhythm reverted to normal in dogs given digoxin by infusion of KCl, plasma potassium concentration was elevated by about 2 mEq/L. Infusion of KCl was not effective in reversing the cardiac arrhythmias caused by ASI-222.

DISCUSSION

We compared the properties of ASI-222, an aminosugar digitalis derivative, and digoxin to bind to and inhibit the activity of partially purified dog heart Na^+,K^+-ATPase and the effect of K^+ on these processes. Additionally, we wished to determine how potassium affects cardiac arrhythmias induced by these agents. Inhibition of myocardial Na^+,K^+-ATPase activity by cardiac glycosides appears to be involved in both the inotropic actions [1, 2, 5, 9] and subsequently the arrhythmic actions of digitalis [20]. Potassium stimulates Na^+,K^+-ATPase activity and also reduces binding of cardiac glycosides to Na^+,K^+-ATPase [1, 11, 13, 26, 27]. Administering KCl has been shown to reverse digitalis cardiotoxicity [21, 22]. ASI-222 has therapeutic potential as an inotropic [19] and antiarrhythmic agent [17]. It possesses a greater cardiotonic potency and therapeutic index than the neutral-sugar derivatives of digitalis [16]. The binding properties of ASI-222 to this enzyme and the effect of potassium, however, have not been studied. Our data indicate that, in the presence of 10 mM K^+, ASI-222 was about 10-fold more potent an inhibitor of cardiac Na^+,K^+-ATPase than digoxin. This finding agrees with our previous findings involving isolated pig brain Na^+,K^+-ATPase [16]. Additionally, we found that there was a difference in the ability of potassium to alter the inhibitory action and binding properties of digoxin and ASI-222 upon this enzyme.

We found that increasing concentrations of potassium in the medium produced parallel shifts of the concentration–inhibition curves for digoxin on Na^+,K^+-ATPase activity to the right. These findings are in concert with our studies of digoxin binding in which increasing potassium concentration reduced the affinity of binding of this drug to the enzyme; that is, the apparent K_D values obtained with 1, 10, and 80 mM potassium were similar to the respective IC_{50} values, suggesting that inhibition of the enzyme by digoxin is closely associated with its binding to this enzyme. The maximum number of binding sites, B_{max}, for digoxin was not affected by different concentrations of potassium and is in the range of values reported by Allen *et al.* [28] for ouabain; they used a protocol similar to ours [29] for their dog heart enzyme preparation.

The influence of potassium on inhibition of enzyme activity by ASI-222 was far less than that observed with digoxin. A 2.5-fold increase in the IC_{50} for ASI-222 was seen when potassium was increased from 1 to 10 mM in the medium. The corresponding increase in IC_{50} for digoxin was 9-fold. In addition, IC_{50} for ASI-222 did not change any further when potassium was increased to 80 mM. The IC_{50} values for ASI-222 were 15- to 30-fold smaller than the K_D values for this agent, suggesting that ASI-222, unlike digoxin, may be binding to sites, all of which may not be functional in terms of inhibition of the enzyme. It is still quite clear, however, that potassium does

Table 2. Effect of KCl infusion on cardiac toxicity to two cardiac glycosides in anesthetized dogs

	Toxic dose of glycoside* (μg/kg)	Total dose of KCl for reversal of toxicity† (mEq/kg)	Plasma $[K^+]$ (mEq/L)	
			Before KCl infusion	At reversal of toxicity
Digoxin	121 ± 17	0.18 ± 0.05 (4/4)	3.40 ± 0.07	5.40 ± 0.66
ASI-222	99 ± 5	No reversal (0/4)	3.54 ± 0.24	No reversal

Values are expressed as means ± SE, N = 4 for both groups.

* Total dose of glycoside producing cardiac toxicity (either 50% occurrence of ventricular ectopic beats within a minute, or evidence of second degree A-V nodal blockade).

† KCl infusion (5 mEq/kg/hr) was started with the development of arrhythmias and maintained until cardiac rhythm reverted to normal (digoxin) or death (ASI-222). Fractions in parentheses are portions of each group which reverted to normal rhythm.

reduce the ability of digoxin to inhibit the enzyme more than it reduces the action of ASI-222. The increase in B_{max} for ASI-222 after addition of potassium probably represents a combination of functional—sites related to inhibition of the enzyme—and non-functional sites. Potassium appears to reveal additional, non-physiological binding sites for this agent. A plot of percent enzyme inhibition by ASI-222 versus percent ASI-222 binding to the enzyme in the presence of 10 mM potassium (Fig. 3) demonstrated that glycoside binding continued after enzyme inhibition was completed. This was not true for digoxin.

Although two classes of binding sites for [^{3}H]ouabain have been reported in cardiac Na^+,K^+-ATPase preparations from neonatal rats [6], guinea pigs [4, 30], isolated canine [5], and chick heart cells [9], there was no indication of multiple binding sites for digoxin or ASI-222 in our dog heart enzyme preparation. The linear profiles of Scatchard plots in the absence and presence of potassium indicated that digoxin binds to a single or homogeneous population of binding sites. Isoenzymes of Na^+,K^+-ATPase with alpha and alpha (+) catalytic subunits have been described for brain tissues by Sweadner [31]. Two forms of this enzyme, one with high and one with low affinity for digitalis glycosides, have been described in other tissues as well [32, 33]. Recently Maixent *et al.* [34] reported K_D values of 3 and 300 nM for ouabain binding sites in microsomal fractions from dog cardiac myocytes. Our K_D values, 26 and 207 nM for digoxin and ASI-222, respectively, in the absence of potassium are in between the high and low affinity K_D values for ouabain binding reported by Maixent *et al.* [34]. Studies from our own laboratory with isolated Ca^{2+}-tolerant dog cardiac myocytes have revealed K_D values of 53 nM and 35 μM for ouabain [5]. Data in the literature for digoxin binding sites are sparse; furthermore, ASI-222 has never been studied for its binding properties to Na^+,K^+-ATPase. Our finding of a single population of binding sites for digoxin and ASI-222, rather than multiple sites, may be explained by different binding properties of different digitalis glycosides. It has been shown by Lichtstein *et al.* [10] by using quantitative computer modelling that rat heart and brain enzymes display two classes of binding sites. However, only ouabain discriminates between the high and low affinity sites (K_D 40 and 2300 nM respectively). Digoxin and digitoxin have nearly identical affinities for both sites (50–71 nM).

We noted that i.v. infusion of KCl reversed cardiac arrhythmias to digoxin in anesthetized dogs. This finding agrees with several previous reports demonstrating the ability of potassium or potassium-sparing diuretics to reverse or reduce the cardiac toxicity of ouabain and acetylstrophanthidin [21, 22, 35]. In contrast, cardiac arrhythmias to ASI-22 are resistant to exogenously administered potassium. If the binding of ASI-222 to a site on Na^+,K^+-ATPase and subsequent inhibition of activity are responsible initially for the inotropic and then for the toxic actions of this agent, only a fraction of these sites appears to be responsible for inhibition of the enzyme. Within the physiological concentration range, potassium does not appear to affect the IC_{50} for ASI-222 as much as it does for digoxin. That is, potassium reduced the ability of digoxin to inhibit the enzyme more than it reduced the action of ASI-222. Potassium chloride does not appear to be an effective antidote for ASI-222 cardiotoxicity produced in healthy myocardium. This finding does not exclude the possibility that, with lower but pharmacologically effective doses of ASI-222, potassium may reverse toxic arrhythmias in the ischemic or failing heart.

The inability of potassium to reverse the cardiac toxicity of ASI-222 may be explained by the chemical property of this compound. As proposed by Akera *et al.* [11, 12], potassium may retard the dissociation of lipid-insoluble agents such as ASI-222 from Na^+,K^+-ATPase by imposing a lipid barrier over binding sites. Lack of change in binding affinity in the presence of 1 and 10 mM potassium supports this theory. Another possibility is that the cardiac toxicity produced by ASI-222 results from its binding to a nonglycoside site, a possibility consistent with the finding that the addition of potassium increases the maximum binding of ASI-222 yet does not reverse the arrhythmias produced by this agent. The effect of ASI-222 on the electrophysiology and the contractility may be dissociated by the nature of its binding to the cardiac membrane. We have shown earlier that ASI-222 differs from digoxin in its effect on the A-V refractory period [17]. Our data cannot adequately explain these speculations, and additional studies are required to determine the

mechanisms of arrhythmias induced by these two agents to explain these findings fully.

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