Cardiac Sodium, Potassium-Adenosine Triphosphatase as a Possible Site of Adriamycin-induced Cardiotoxicity

Larry P. Solomonson and Paul R. Halabrin

Department of Biochemistry, University of South Florida, College of Medicine, Tampa, Florida 33612

ABSTRACT

Adriamycin was tested as a possible inhibitor of cardiac sodium-potassium-activated adenosine triphosphatase (Na-K-ATPase). At concentrations of $10^{-4}$ M and lower, Adriamycin had no effect upon either ouabain-sensitive (Na-K-ATPase) or ouabain-insensitive adenosine triphosphatase activity in homogenates and microsomal fractions of cardiac tissue from several different species. Adriamycin inhibited adenosine triphosphatase activity at a concentration of $10^{-3}$ M, but this was due to the inhibition of ouabain-insensitive adenosine triphosphatase rather than to inhibition of Na-K-ATPase. Under no condition was an inhibition of Na-K-ATPase activity by Adriamycin observed. These conditions included preincubation of the enzyme with Adriamycin, chelation of Ca$^{2+}$, addition of reduced nicotinamide adenine dinucleotide phosphate, and variation of buffer and pH. It was concluded that Na-K-ATPase is not a likely site of Adriamycin-induced cardiotoxicity.

INTRODUCTION

The anthracycline drug Adriamycin is one of the most effective antitumor agents presently available for use in cancer chemotherapy. The therapeutic use of this drug, however, is limited due to a severe, cumulative cardiotoxicity which is frequently associated with long-term Adriamycin therapy.

Adriamycin appears to exert its antitumor effect by intercalation with DNA (17, 18), but its cardiotoxic effect is apparently due to some other action of the drug (8). Several hypotheses have been proposed to explain the cardiotoxicity of Adriamycin, including its effect on mitochondrial function (4), lipid peroxidation (14), and calcium flux (3, 16), but none has been unequivocally demonstrated. Recently, Gosálvez et al. (5) suggested that Adriamycin cardiotoxicity may be a digitalis type of cardiotoxicity, based on their finding that Adriamycin inhibits Na-K-ATPase activity at concentrations as low as $10^{-11}$ M. These concentrations are several orders of magnitude lower than inhibitory concentrations of the cardiac glycosides (digitalis) which are specific inhibitors of Na-K-ATPase. We were interested in confirming these results in order to further explore the molecular basis for the extreme sensitivity of this important transport enzyme to Adriamycin.

In this report, we describe our efforts to confirm the results of Gosálvez et al. (5). Our results have led us to the conclusion that Adriamycin is not an inhibitor of cardiac Na-K-ATPase but rather an inhibitor of ouabain-insensitive ATPase (Mg-ATPase) at higher concentrations.

MATERIALS AND METHODS

Materials. All chemicals were of the highest grade available from commercial sources. Yeast glucose-6-phosphate dehydrogenase (type XII) and superoxide dismutase (type I) were obtained from Sigma Chemical Co., St. Louis, Mo. Adriamycin was obtained either from Aldrich Chemical Co., Milwaukee, Wis., or from Sigma. Solutions of Adriamycin in water were prepared fresh and were protected from light.

Preparation of Cardiac Na-K-ATPase. Several different methods were used to prepare Na-K-ATPase from rat, monkey, dog, or rabbit cardiac tissue (fresh or frozen). In all cases, the specific activities and degree of sensitivity to the cardiac glycoside ouabain, were within reported ranges (1, 5). All steps were carried out at 0–4°.

Crude homogenate was prepared by suspending minced heart tissue in 0.25 mM sucrose-10 mM hydroxyethylpiperazine-N'-2-ethanesulfonic acid-NaOH buffer (pH 7.5) and homogenizing gently with a glass-Teflon homogenizer. This homogenate was passed through 4 layers of cheesecloth, and the filtrate was centrifuged at 500 x g for 5 min. The supernatant was then used as the crude homogenate fraction. Microsomal fractions were prepared by the method of Auditore and Murray (1) or exactly as modified by Gosálvez et al. (5). These latter 2 preparative procedures involve extraction of the heart tissue with a 0.1% solution of sodium deoxycholate at either pH 6.0 (1) or pH 7.0 (5). Alternatively, the microsomal fraction was prepared from tissue homogenates which were extracted with 0.5 M KCl (7) instead of detergent.

Enzyme Assays. ATPase activity was measured at 37° by determining the rate of P$_i$ released from ATP in the presence and absence of enzyme (10). In all cases, the total amount of P$_i$ released corresponded to the hydrolysis of less than 10% of the total amount of ATP. Under these conditions, the rate of release was linear with time and was directly proportional to the amount of enzyme added. Several different modifications of the assay mixture involving changes in pH and buffer were tested in the course of these studies. Our standard assay mixture contained 30 mM hydroxyethylpiperazine-N'-2-ethanesulfonic acid-NaOH (pH 7.4), 130 mM NaCl, 20 mM KCl, 3 mM MgCl$_2$, and 3 mM ATP. The assay mixture described by Gosálvez et al. (5) was composed of 80 mM imidazole-HCl (pH 6.7), 140 mM NaCl, 14 mM KCl, 3 mM MgCl$_2$, and 3 mM ATP. A mixture identical to the latter mixture except with a pH of 7.4 was also tested. Assays were run in the presence or absence of 1 mM ouabain plus the indicated concentrations of Adriamycin. Na-K-ATPase activity is completely inhibited by 1 mM ouabain. The activity of Na-K-ATPase can therefore be com-

Received February 4, 1980; accepted November 4, 1980.

1 This research was supported in part by a grant from the American Heart Association, Florida Affiliate.

2 To whom requests for reprints should be addressed, at the Department of Biochemistry, University of South Florida College of Medicine, 12901 North 30th Street, Tampa, Fla. 33612.

3 Supported by an R. G. Thompson Fellowship from the American Cancer Society.

Deceased.

Received February 4, 1980; accepted November 4, 1980.
puted from the difference in activity in the presence and absence of 1 mM ouabain. All measurements were done in triplicate. Values generally varied less than 5% between replicate samples. Blanks and standards which contained no enzyme were run for each reaction mixture. Where indicated, an NADPH-regenerating system consisting of 10 mM glucose-6-phosphate, 2 mM NADPH, and 5 units of glucose-6-phosphate dehydrogenase per ml was included in the ATPase assay mixture to test for the effect of NADPH. One unit of glucose-6-phosphate dehydrogenase is the amount which will catalyze the oxidation of 1 μmol of NADPH per min. Protein was determined by the method of Lowry et al. (11).

In some cases, latent Na-K-ATPase was activated by preincubation of the enzyme preparations with low concentrations of sodium dodecyl sulfate in the presence of ATP. In some cases, the observed inhibition of total ATPase activity by Adriamycin was significantly greater than that observed for 1 mM ouabain, a specific inhibitor of Na-K-ATPase. Na-K-ATPase is completely inhibited by 0.1 mM ouabain; thus, 1 mM represents a large excess of this inhibitor. If Adriamycin also acts on Na-K-ATPase, there should be no additive effect of the 2 inhibitors at these concentrations. If, on the other hand, Adriamycin acts on a different ATPase (non-ouabain sensitive), the inhibition of total ATPase activity by a combination of the 2 inhibitors should be additive. This was found to be the case which led to the conclusion that Adriamycin does not inhibit Na-K-ATPase activity under these conditions. The inhibition of ATPase activity by 1 mM Adriamycin could not be reversed by a procedure in which the Adriamycin-treated microsomal fraction was diluted 10-fold with Adriamycin-free medium, followed by centrifugation of the diluted mixture for 1 hr at 100,000 × g and resuspension of the sedimented membrane particles in Adriamycin-free medium. The Adriamycin-treated particles were bright red after this procedure, indicating substantial association of Adriamycin with the membrane particles. Microsomal fraction which was not treated with Adriamycin was not affected by this treatment. Therefore, the inhibition of ATPase activity by Adriamycin appears to be irreversible. No significant inhibition of total ATPase activity by Adriamycin was observed between 10⁻⁴ and 10⁻¹ M.

We attempted to find conditions under which cardiac Na-K-ATPase would be inhibited by low concentrations of Adriamycin. For these experiments, ATPase activity of rabbit heart microsomal fraction (5) was measured in the presence and absence of 1 mM ouabain, 10⁻⁸ M Adriamycin, or 10⁻⁴ M Adriamycin. No significant inhibition of cardiac Na-K-ATPase by these concentrations of Adriamycin was observed under various conditions. For example, the possibility that inhibition of Na-K-ATPase by Adriamycin may be a time-dependent reaction was tested by preincubation of the enzyme for up to 2 hr at 37° before starting the enzyme assay by addition of ATP. Several different preparations of enzyme from different animal species (see "Materials and Methods"), ranging from crude homogenate to partially purified membrane fractions, were also tested for sensitivity to Adriamycin. The same results (negative) were obtained whether enzyme was prepared from fresh tissue or from tissue which had been stored frozen. Likewise, buffer type and pH of the assay mixture (see "Materials and Methods") were not factors in the sensitivity of the enzyme to Adriamycin. The enzyme was preincubated for 1 hr with Adriamycin and 1 mM ethylene glycol bis(β-aminoethyl ether)-N,N',N",N"'-tetraacetic acid. Handa and Sato (6) and Bachur et al. (2) have reported that, in microsomal preparations exposed to anthracyclines such as Adriamycin, there is increased production of superoxide radicals which leads to lipid peroxidation (14). This superoxide radical production is dependent on NADPH (2, 6). Hoxum and Fried (9) have recently shown that Na-K-ATPase from rat brain can be slowly and irreversibly inactivated by exposure to superoxide radicals. This inactivation could be prevented by further addition of superoxide dismutase. In order to facilitate any Adriamycin-dependent superoxide radical formation, we preincubated the enzyme for various times up to 2 hr with Adriamycin and an NADPH-regenerating system consisting of NADPH, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase (see "Materials and Methods"). One set of incubation mixtures also contained superoxide dismutase at a concentration of 50 units/ml (12). No effect of Adriamycin was observed either in the presence or absence of the NADPH-regenerating system or in the presence or absence of superoxide dismutase. In the course of these studies, we used 3 different lots of Adriamycin obtained from 2 different suppliers. Melting point

### RESULTS

As shown in Table 1, 1 mM Adriamycin inhibits ATPase activity in the microsomal fraction as well as in crude homogenates of rabbit cardiac tissue. In some cases, the observed inhibition of total ATPase activity by Adriamycin was significantly greater than that observed for 1 mM ouabain, a specific inhibitor of Na-K-ATPase. Na-K-ATPase is completely inhibited by 0.1 mM ouabain; thus, 1 mM represents a large excess of this inhibitor. If Adriamycin also acts on Na-K-ATPase, there should be no additive effect of the 2 inhibitors at these concentrations. If, on the other hand, Adriamycin acts on a different ATPase (non-ouabain sensitive), the inhibition of total ATPase activity by a combination of the 2 inhibitors should be additive. This was found to be the case which led to the conclusion that Adriamycin does not inhibit Na-K-ATPase activity under these conditions. The inhibition of ATPase activity by 1 mM Adriamycin could not be reversed by a procedure in which the Adriamycin-treated microsomal fraction was diluted 10-fold with Adriamycin-free medium, followed by centrifugation of the diluted mixture for 1 hr at 100,000 × g and resuspension of the sedimented membrane particles in Adriamycin-free medium. The Adriamycin-treated particles were bright red after this procedure, indicating substantial association of Adriamycin with the membrane particles. Microsomal fraction which was not treated with Adriamycin was not affected by this treatment. Therefore, the inhibition of ATPase activity by Adriamycin appears to be irreversible. No significant inhibition of total ATPase activity by Adriamycin was observed between 10⁻⁴ and 10⁻¹ M.

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### Table 1

Inhibition of cardiac ATPase activity by Adriamycin and ouabain

<table>
<thead>
<tr>
<th>Enzyme fraction</th>
<th>Sodium dodecyl sulfate pretreatment</th>
<th>Inhibitor</th>
<th>% of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>No</td>
<td>Ouabain</td>
<td>5.9 ± 1.2a</td>
</tr>
<tr>
<td>Homogenate</td>
<td>No</td>
<td>Adriamycin</td>
<td>37.0 ± 4.3</td>
</tr>
<tr>
<td>Homogenate</td>
<td>No</td>
<td>Ouabain plus adriamycin</td>
<td>43.2 ± 2.9</td>
</tr>
<tr>
<td>Homogenate</td>
<td>Yes</td>
<td>Ouabain</td>
<td>32.5 ± 3.0</td>
</tr>
<tr>
<td>Homogenate</td>
<td>Yes</td>
<td>Adriamycin</td>
<td>36.8 ± 3.3</td>
</tr>
<tr>
<td>Homogenate</td>
<td>Yes</td>
<td>Ouabain plus adriamycin</td>
<td>66.9 ± 4.7</td>
</tr>
<tr>
<td>Microsomal</td>
<td>No</td>
<td>Ouabain</td>
<td>47.6 ± 1.3</td>
</tr>
<tr>
<td>Microsomal</td>
<td>No</td>
<td>Adriamycin</td>
<td>54.0 ± 1.5</td>
</tr>
<tr>
<td>Microsomal</td>
<td>No</td>
<td>Ouabain plus adriamycin</td>
<td>98.4 ± 1.2</td>
</tr>
</tbody>
</table>

a. Enzyme fractions were prepared from fresh rabbit cardiac tissue. Homogenate was prepared as described in "Materials and Methods.” Microsomal fraction was prepared according to the method of Auditore and Murray (1) as modified by Godlewicz et al. (6).

b. Homogenate was preincubated, where indicated, with sodium dodecyl sulfate as described in "Materials and Methods” in order to activate latent Na-K-ATPase before assay for ATPase activity.

c. Final concentration of each indicated inhibitor in the assay mixture was 1 mM.

d. Mean ± S.D. of total ATPase activity inhibited by the indicated inhibitor.
and absorption maxima of Adriamycin obtained from the sources agreed with reported values (13). Thus, it is unlikely that the observed insensitivity of cardiac Na-K-ATPase to Adriamycin is due to contaminants or to inactivation of the Adriamycin used in these studies.

DISCUSSION

Even at high concentrations of Adriamycin, the observed inhibition of ATPase activity was due largely, if not entirely, to inhibition of non-ouabain-sensitive ATPase. This inhibition is unlikely to be of pharmacological importance, since it was observed only at relatively high (1 mM) concentrations of Adriamycin. We are thus led to the conclusion that Na-K-ATPase is not a likely site for Adriamycin-induced cardiotoxicity as had been suggested from the results of Gosálvez et al. (5). Other data presented by Gosálvez et al. (5) are also consistent with findings that Na-K-ATPase is not the site of the cardiotoxic action of Adriamycin. They found that the apparent inhibition of Na-K-ATPase by Adriamycin could be completely prevented by 20 μM Ca²⁺, which is well below serum levels of Ca²⁺. The apparent inhibition by Adriamycin was also lost upon further purification of the enzyme. Furthermore, Adriamycin did not inhibit sodium transport, the major physiological function of Na-K-ATPase, even at high (2 mM) concentrations of the drug (5). These results support our conclusion that inhibition of Na-K-ATPase by Adriamycin is not the basis for the cardiotoxicity of this drug.

REFERENCES

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