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Inhibition of Sodium-Potassium-activated Adenosine 5'-Triphosphatase and Ion Transport by Adriamycin[1]

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ABSTRACT

The antitumor antibiotic Adriamycin is a potent inhibitor of the sodium-potassium-activated adenosine triphosphatase of native heart microsomes. Adriamycin also inhibits potassium transport (although not sodium transport) in slices of kidney cortex. The effects on both the adenosine triphosphatase and ion transport are markedly reduced by Ca^{2+}, probably by chelation of this metal by Adriamycin. These effects could provide a basis for explaining the Adriamycin cardiotoxicity as a digitalis-type toxicity.

INTRODUCTION

The antitumor antibiotic Adriamycin (8) is presently considered to be a very effective and useful chemotherapeutic agent in the treatment of many human tumors (6). However, its extensive use at doses adequate for effective antitumor therapy is restricted by the appearance of a severe cardiotoxicity, which can be lethal at cumulative doses in excess of 500 mg/sq m (5).

A number of hypotheses have been proposed to explain the mechanism of cardiotoxicity with Adriamycin (1, 4, 10, 20, 22), but none has yet been demonstrated. The fact that Adriamycin has a glycosidic structure which resembles some glycosides with cardiotoxic effect and the fact that rabbit hearts with cardiomyopathy induced by chronic intoxication with Adriamycin present very marked alterations in sodium and potassium content (23) prompted us to study the effect of this drug on Na-K-dependent ATPase and ion transport. We have found that Adriamycin is a very potent inhibitor of the Na-K-ATPase activity of rabbit heart native microsomes in the absence of calcium and that Adriamycin inhibits potassium transport in kidney slices. These effects could be related to the cardiotoxic effect of the drug.

A preliminary report of these results has been presented (11, 30).

MATERIALS AND METHODS

Isolation and Assay of Rabbit Heart Na-K-ATPase. The preparation used was essentially the rabbit heart homogenate described by Auditore and Murray (3), except that the freezing step was omitted. Four rabbit hearts were homogenized in 150 ml of a medium containing 0.25 M sucrose, 20 mM histidine, 5 mM EDTA, and 0.1% deoxycholate (pH 7.0, 0°). The homogenate was centrifuged at 9000 × *g* for 20 min. The supernatant, free of mitochondria and nuclear debris, was centrifuged at 52,000 × *g* for 1 hr at 4°. The supernatant was discarded, and the pellet was washed twice with 0.32 M sucrose plus 1 mM EDTA before being collected in 0.3 M sucrose. This preparation had a mean Na-K-dependent ATPase of 0.2 μmol ATP per min per mg protein and a mean sensitivity to ouabain of 50%. The enzyme preparation was made daily and was used fresh.

The enzyme activity was assayed after the incubation of the enzyme (0.1 mg protein per ml) with the drug for 20 min in a shaking bath at 37°. The incubation medium contained 80 mM imidazole, 3 mM Mg^{2+}, 3 mM ATP, 140 mM Na^+, and 14 mM K^+ (pH 6.7). At the end of the incubation, samples were taken for the assay of P_i. The phosphate was determined as described specifically for ATPase measurements by Post and Sen (24).

The specific activity of the Na-K-ATPase in the preparation (μmol ATP hydrolyzed per min per mg protein) was determined as the difference between the total activity in the presence of 140 mM sodium plus 14 mM phosphate and the activity in the presence of sodium and phosphate plus 0.1 mM ouabain. The sensitivity to Adriamycin was expressed as the percentage of this specific activity of the Na-K-ATPase remaining in the presence of each concentration of the drug. The protein concentration of the enzyme preparation was measured as described by Lowry *et al.* (19). The drug was freshly dissolved in water for each experiment and was kept in the dark.

Measurement of Ion Transport in Tissue Slices. Male albino rabbits weighing approximately 2.5 kg were killed by a blow on the neck. A single kidney was immediately dissected out. The cortex was sliced in a Petri dish cooled with ice using a razor blade guided by a glass slide. The slices were then preincubated for 90 min at 1° in Ringer's solution (161 mM Na^+, 5 mM K^+, 1 mM Mg^{2+}, 151 mM Cl^-, 1 mM SO_4^{2-}, 10 mM phosphate, 1.5 mM Ca^{2+}, and 20 mM glucose) in order to permit the cells to lose K^+ and gain Na^+ (7, 21). After the first 30 min of cold preincubation, the slices were distributed over fresh portions of an appropriate Ringer's solution that was contained in reaction flasks of the Gilson differential respirometer; each flask contained 3 ml of medium and 100 to 150 mg, wet weight, of slices and stood in an ice bath. The center wells of the flasks contained 0.3 ml 5 N KOH. The inhibitors under study were also added to the medium at this point. Five min before the end of the preincubation period, the vessels were attached to the manometers and gassed with O_2. Sample slices were taken to determine total Na^+ and K^+ content of the tissue after preincubation, and the remaining flasks were lowered

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into the bath, which was maintained at 38° for the experimental incubation (60 min). During the period at 38°, the slices reaccumulated K^+ and extruded Na^+ by a mechanism which is dependent upon respiration (21). The net difference in total Na^+ and K^+ content between samples taken before and after incubation at 38° is a measurement of the activity of the Na^+- and K^+-transporting system (9, 29). The Na^+ and K^+ content of the tissue slices was determined by atomic absorption spectrophotometry (Perkin Elmer 303) as described previously (28).

Arterially Perfused Rabbit Interventricular Septum Preparations. This preparation of a perfused heart has been described previously in detail (17), and with the septal tissue mounted and perfused as described it was possible to record continuously and simultaneously: (*a*) the frequency of contraction with the rate controlled by external stimulation; (*b*) force; and (*c*) isometric tension with electronic differentiation of the tension trace to allow direct recording of *dP/dt*. All experiments were done at temperatures between 25 and 28.5°. The perfusion rate was 1.2 to 1.5 ml/g tissue per min and was constant in each experiment. The ionic composition of the perfusate was as follows (concentrations in mmol/liter): NaCl, 142; KCl, 4; $CaCl_2$, 1.5; NaH_2PO_4, 0.53; $MgCl_2 \cdot 6H_2O$, 1.0; and glucose, 5.56. The perfusate contained washed dog RBC at a hematocrit reading of 20%, and immediately prior to use it was equilibrated to 98% O_2:2% CO_2. The oxygen content was approximately 0.13 ml O_2 per ml, and the pH was 7.3.

RESULTS

Chart 1 shows the inhibition of the Na-K-dependent ATPase activity of rabbit heart native microsomes by increasing concentrations of Adriamycin. Adriamycin seems to be a very potent inhibitor acting at a wide range of concentrations, as do other glycosides which are inhibitors of Na-K-ATPase (25). The inhibition was lost upon purification of the enzyme with deoxycholate or NaI, indicating that, for inhibition with Adriamycin, the integrity of the membrane is required. As the Na-K-ATPase is widely considered to be the enzyme system of the plasma membrane responsible for the active transport of Na^+ and K^+ (26), we undertook the study of the effect of Adriamycin on ion transport in kidney slices.

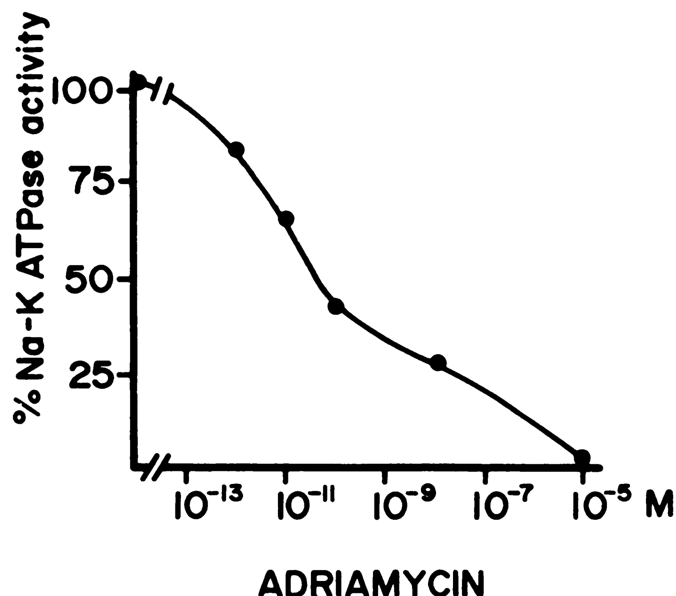

Chart 1. Effects of increasing concentrations of Adriamycin on the Na-K-dependent ATPase activity of a rabbit heart microsomal preparation. One hundred % activity represents a specific activity of 0.2 µmol ATP hydrolyzed per min per mg protein. Each experimental *point* is the mean of 2 determinations.

Initial experiments with kidney slices were conducted in Ringer's solution buffered with Tris-HCl or phosphate to pH 7.4 and containing 1.25 mM Ca^{2+}, since these conditions are known to support ion transport in this preparation. As seen in Table 1, in both Tris-buffered and phosphate-buffered Ringer's medium, the slices lost 50 to 57% of their K^+ content and more than doubled their Na^+ content during incubation at 1°. These changes were reversed to a considerable extent during subsequent incubations at 38°, and ouabain (200 µM) completely prevented the recovery. In contrast, Adriamycin had no effect on the transport even at concentrations as high as 500 µM.

The unexpected insensitivity of ion transport in kidney slices to Adriamycin led us to modify the incubation medium by omitting Ca^{2+}, since it is known that Adriamycin is a potent chelator for Ca^{2+} (31), and by reducing the pH of the medium to 6.9, the optimum pH for Adriamycin inhibition of the Na-K-ATPase. It is known that the absence of Ca^{2+} from the incubation medium increases the "leakiness" of kidney cells to Na^+ and K^+ (15), and we found that, in the absence of Ca^{2+}, the net transport of Na^+ and K^+ is reduced by about 25%. The remaining transport, however, represents a large movement of ions to permit a reliable study. Chart 2 illustrates the effect of Adriamycin on Na^+ and K^+ transport in kidney slices incubated in the Ca^{2+}-free, phosphate-buffered Ringer's solution at pH 6.9. There appears to be a small inhibition of K^+ reaccumulation at 0.1 mM Adriamycin, while at concentrations of 0.2 mM and above the effects were statistically significant ($p > 0.01$ in all cases) when tested by analysis of variance and the variance

Table 1

Net movements of Na^+ and K^+ in slices of rabbit kidney cortex and the effects of Adriamycin and ouabain in media containing 1.25 mM Ca^{2+} at pH 7.4

Incubation	Ion content (mmol/kg dry wt) K^+	Na^+
Tris-buffered Ringer's solution ($n = 2$)		
Fresh tissue	328	325
90 min at 1°	124	679
Plus 60 min at 38°, control	254	373
With ouabain (200 µM)	84	681
With Adriamycin (50 µM)	250	405
With Adriamycin (100 µM)	245	392
Phosphate-buffered Ringer's solution ($n = 13$)		
90 min at 1°, control	166 ± 17[a]	801 ± 50
With Adriamycin (500 µM)	170 ± 13	670 ± 51
Plus 60 min at 38°, control	303 ± 17	483 ± 50
With Adriamycin (500 µM)	301 ± 16	577 ± 62

[a] Mean ± S.E.

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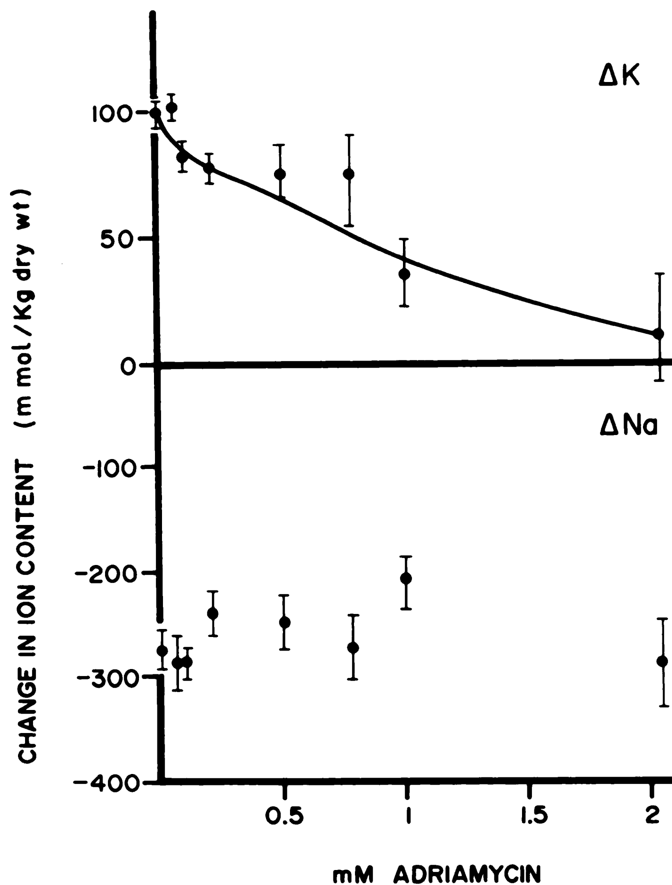

Chart 2. Change in Na^+ and K^+ content upon rewarming kidney slices in calcium-free medium in the absence or presence of increasing concentrations of Adriamycin. Adriamycin inhibits the accumulation of K^+ but does not significantly affect the extrusion of Na^+.

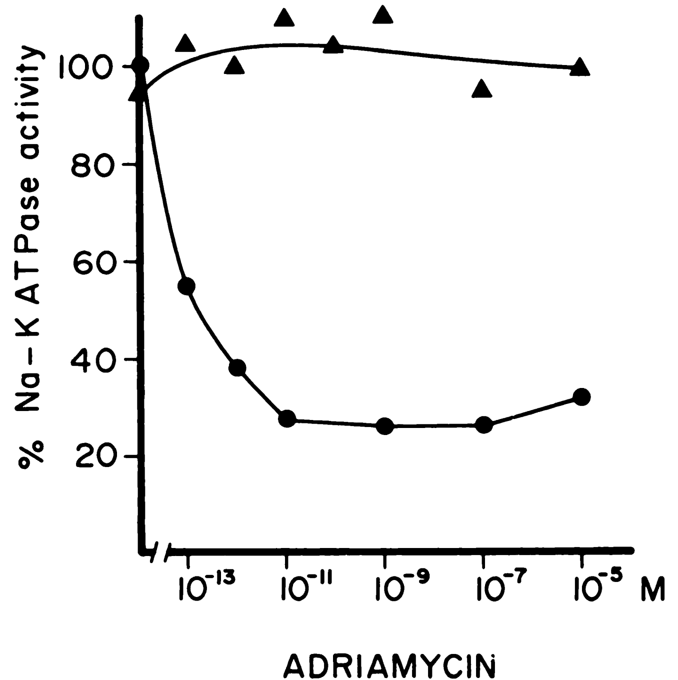

Chart 3. Effects of increasing concentrations of Adriamycin on the activity of rabbit heart Na-K-ATPase measured in the absence (●) or in the presence (▲) of 20 μM calcium. Each experimental *point* represents the mean of 2 determinations. Adriamycin inhibits the Na-K-dependent ATPase activity only in the absence of calcium. One hundred % activity represents a specific activity of 0.2 μmol ATP hydrolyzed per min per mg protein.

ratio (*F*) test. In the presence of 2 mM Adriamycin, no significant reaccumulation of K^+ occurred; in contrast, the net extrusion of Na^+ was not significantly reduced by Adriamycin at any of the concentrations tested.

In view of the effect of the presence of calcium in the medium on the sensitivity of ion transport to Adriamycin, we studied the effect of Ca^{2+} on the inhibition of the Na-K-ATPase by this drug. Chart 3 shows the effect of increasing concentrations of Adriamycin on rabbit heart Na-K-ATPase assayed in the presence or absence of 20 μM Ca^{2+}. It can be seen that in the presence of Ca^{2+} there was no inhibition. These results are interpreted to indicate that Adriamycin possibly forms a calcium-Adriamycin complex and that this complex is inactive in the enzyme.

Attempts were made to detect inhibition of ion transport by Adriamycin in slices of rabbit heart. However, in our hands the rabbit heart slices in the absence of calcium or in the presence of low calcium failed to transport ions. The transport in the presence of 1 mM calcium was totally insensitive to Adriamycin. As an alternative way to define the effects of Adriamycin in heart tissue, the drug was perfused intraarterially in the rabbit interventricular septum preparation (17) to define its effects on the force of contraction of the heart and on *dP/dt*. Chart 4 shows that Adriamycin at a concentration of 20 μg/ml of perfusate elicited an increase in force and *dP/dt*, thus defining a positive inotropic effect similar to that which is found with ouabain in this preparation. Adriamycin at 70 μg/ml of perfusate elicited a toxic negative inotropic effect with depression of heart force and *dP/dt* after a transient rapid increase.

DISCUSSION

The results show that Adriamycin is a potent inhibitor of the Na-K-ATPase activity of rabbit heart native microsomes. Preliminary results showed that other Na-K-ATPases, such as mouse heart, Ehrlich ascites tumor, or shark salt gland Na-K-ATPase, are also inhibited in the native state. The pattern of the inhibition of the ATPase has a wide-range concentration dependency. These characteristics, which are also seen in the inhibition of the enzyme by ouabain (3), could be due to the presence of the enzyme in various conformational states, each with a different sensitivity to the inhibitor (2, 13). The finding that the inhibition with Adriamycin is lost upon purification of the enzyme is puzzling. It seems that the integrity of the membrane is required for the inhibition with Adriamycin. Solie and Yuncker (27) also ascribed to a general membrane effect, the stimulation by Adriamycin of the passive influx of sodium in frog skin.

The inhibition of ion transport in kidney slices with Adriamycin is seen at much higher concentrations (0.2 to 2 mM) than the effect on the ATPase and only if the transport is assayed in the absence of calcium. It is tempting to attribute the rather low sensitivity of transport activity in slices, as compared to that of the ATPase, to the presence of varying quantities of cell Ca^{2+} and, therefore, to the

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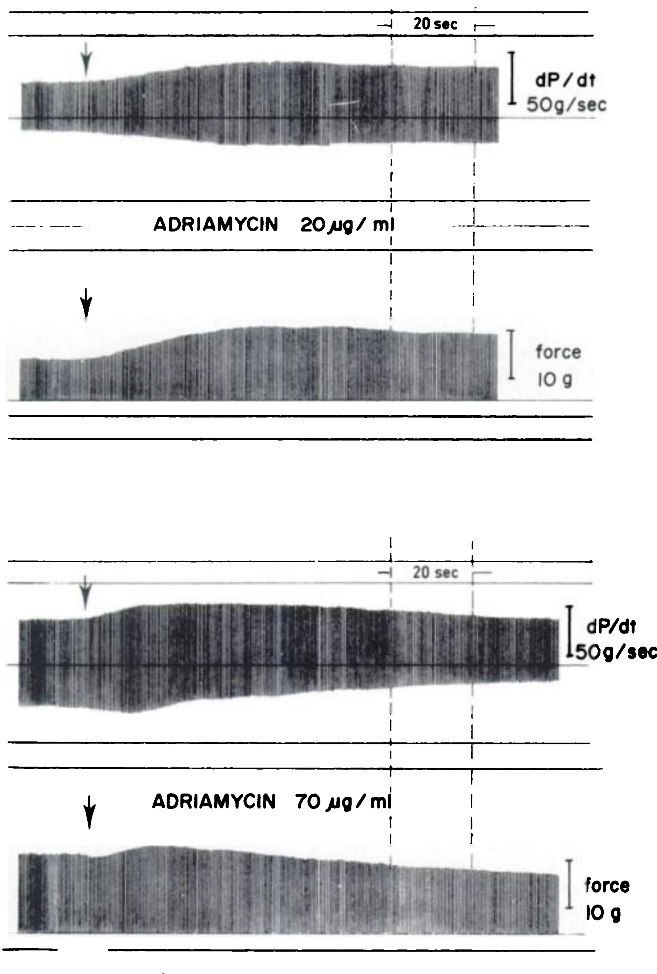

Chart 4. Effects of Adriamycin on force and *dP/dt* in the arterially perfused rabbit interventricular septum preparation. In the *upper 2 traces*, Adriamycin produces an inotropic positive effect and in the *lower traces* it produces an inotropic negative effect when added to the perfusate at the indicated concentration.

reduction in availability of the unchelated active form of Adriamycin. Furthermore, Adriamycin is largely bound to all tissue constituents, which may in turn reduce further the availability of the drug for the active sites in the membrane. However, it is difficult to make a comparison of the effect of the drug in an enzyme in rabbit heart with the effect of ion transport in kidney. Although we did not show an effect on cardiac ion transport, preliminary results have shown that potassium transport is also inhibited with Adriamycin in rabbit muscle (soleus) or in Ehrlich ascites cells at a 200 µM concentration of the drug.

An intriguing result is the finding that Adriamycin causes a nearly complete inhibition of K^+ reaccumulation while failing to affect Na^+ extrusion. Although an ouabain-insensitive mechanism for Na^+ extrusion has been reported in kidney cells (14), our results failed to show evidence of this mechanism in either the presence or the absence of calcium. An alternative explanation for the lack of effect of Adriamycin on Na^+ transport may be that the drug has uncoupled the Na^+ transport aspects of the system from its dependency on K^+. This would represent a novel inhibitory effect in ion transport.

The effects of Adriamycin on the rabbit heart Na-K-ATPase and on K^+ transport could provide a possible explanation for the cardiotoxicity of the drug as a digitalis-like effect, especially since we have found that Adriamycin is able to elicit an inotropic positive and an inotropic negative effect in perfused heart septum. Langer (16, 18) has demonstrated that the positive inotropic effect (cardiotonic) of ouabain as well as its cardiotoxic effect (negative inotropic effect) in rabbit heart are related to the inhibition of Na-K-ATPase and ion transport. However, our results do not as yet exclude any of the other possible mechanisms of cardiac toxicity of Adriamycin (1, 4, 10, 20, 22). Lipid peroxidation (20) may be one of the reasons for the toxicity of Adriamycin and may be the reason for the inhibition of Na-K-ATPase, since the enzyme is well known to be lipid dependent and the drug is incubated with the membrane preparation for 20 min at 37°. In any event, based on the results of this paper, we have prepared a triferric derivative of Adriamycin made by chelation at neutral pH which is inactive in the Na-K-ATPase. This derivative, which has been named quelamycin, appears not to be cardiotoxic and to have less general toxicity while maintaining antitumoral activity (12). These results would support the theory that the inhibition of the Na-K-ATPase is related to the cardiotoxicity.

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