THE CARDIOTOXIC ANTIBIOTIC DOXORUBICIN INHIBITS THE Na^+/Ca^{2+} EXCHANGE OF DOG HEART SARCOLEMMAL VESICLES

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1. Introduction

The antitumoral anthracycline antibiotic doxorubicin (adriamycin) has cardiotoxic side effects that have been related to disturbances in the intracellular metabolism of Ca^{2+}. A number of authors have reported alterations in mitochondrial Ca^{2+} transport in heart, ranging from inhibition of uptake by mitochondria upon exposure to doxorubicin in vitro [1,2] to stimulation of uptake in rabbits chronically intoxicated with the antibiotic [3]. Substantial inhibitory effects on mitochondrial Ca^{2+} uptake were seen at concentrations of doxorubicin in excess of 100 μM, and were not seen, or seen only at extremely high concentrations of the antibiotic, when the process was energized by ATP. The other Ca^{2+} transporting intracellular organelle in heart, the sarcoplasmic reticulum, was not affected by doxorubicin [1]. In heart cells, Ca^{2+} fluxes are controlled also by two plasma membrane (sarcolemma) pumping systems, a specific ATPase [4], and a Na^+/Ca^{2+} exchange system [5,6]. The Ca^{2+} disturbances induced by doxorubicin could thus conceivably be mediated (also) by the impairment of either one of these two pumping functions, or of both. The involvement of sarcolemma is indicated by the finding [7] of reduced exchangeability of Ca^{2+} in doxorubicin-treated isolated guinea pig atria. In the present article, we wish to report on the inhibition of the Na^+/Ca^{2+} exchange in isolated dog heart sarcolemmal vesicles by low concentrations of doxorubicin. The finding may have important pharmacological implications, and opens new possibilities in the study of the Na^+/Ca^{2+} exchanger. Doxorubicin is the first known specific inhibitor of this exchange system.

2. Materials and methods

Dog heart sarcolemmal vesicles were prepared by a slight modification [8] of the procedure of Jones et al. [9]. After suspension in 160 mM NaCl, 20 mM Hepes, pH 7.4 (Na-medium) at a protein concentration of approximately 5 mg ml^{-1}, the vesicles were immediately frozen in liquid nitrogen and stored at −80°C. The vesicle preparations were routinely enriched about 100 fold in sarcolemmal markers as compared to the initial homogenate. Ca^{2+} transport was measured either with the Ca^{2+}-sensitive dye Arsenazo III [10] monitored at 685–660 nm in an Aminco DW-2 spectrophotometer (mitochondrial Ca^{2+} fluxes [11] and sarcolemmal Na^+/Ca^{2+} exchange [8]), or isotopically (ATP-supported Ca^{2+}-pump in heart sarcolemma [4]).

Control experiments showed that the response of Arsenazo III to $[Ca^{2+}]$changes was not altered by the addition of doxorubicin up to a concentration of 100 μM. When Ca^{2+} binding to the sarcolemmal vesicles was measured, KCl-preloaded sarcolemmal vesicles were diluted in 160 mM KCl, 20 mM Hepes, 0.5 μM free $^{45}Ca^{2+}$ at 37°C, and 100 μl-aliquots were subsequently withdrawn and filtered (millipore filters, pore diameter: 0.22 μm). The filters were washed with 2 ml of 160 mM TrisCl, pH 7.4 and then assayed for $^{45}Ca^{2+}$ radioactivity.

Arsenazo III was obtained from Fluka AG, Buchs, Switzerland. Valinomycin was from Sigma. Doxorubicin and its 4′-epimer were obtained from Farmitalia, Milano, Italy and dissolved in H_2O. The solutions were prepared on the day of the experiment, and protected from light.

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3. Results and discussion

A number of experiments performed on isolated heart mitochondria have failed to detect significant alterations of either the uptake of Ca^{2+} or of its Na^+-induced release, in the presence of concentrations of doxorubicin ranging between 5 and 80 μM. In the same concentration range doxorubicin had no effect on the Ca^{2+}-pumping ATPase of the plasma membrane and on the associated Ca^{2+} uptake, indicating that the drug did not alter the passive permeability of the sarcolemmal membrane. By contrast, it depressed very markedly the initial velocity of the sarcolemmal Na^+/Ca^{2+} exchange, 50% inhibition being observed at 10 μM. At 40 μM, the inhibition reached 80% (table 1, and fig.1). The inhibition was not prevented by increasing the external level of free Ca^{2+}, indicating that the inhibition was not of the competitive type. One interesting derivative of doxorubicin is the 4′-epimer, which has proven to be less cardiotoxic in vivo, and in cells grown in vitro [12], than the parent compound. Table 1 shows that the 4′-epimer has a less evident inhibitory effect on the Na^+/Ca^{2+} exchange.

It was of interest to study whether the inhibition reflected the (tight) binding of doxorubicin to the exchanger, or to the membrane domain surrounding

Table 1
Effect of doxorubicin and of its 4′-epiderivative on Ca^{2+} influx into heart sarcolemmal vesicles through the Na^+/Ca^{2+} exchanger

Additions	Initial rate of Ca^{2+} uptake (nmol Ca^{2+} per mg protein per s)	
None	9.8	100%
Doxorubicin		
5 μM	7.9	80.6%
10 μM	4.2	42.8%
20 μM	2.5	25.5%
40 μM	1.8	18.3%
80 μM	1.5	15.3%
4′-epidoxorubicin		
10 μM	9.5	96.9%
20 μM	5.1	52.0%
40 μM	3.9	39.8%

Experimental conditions as in fig.1. The initial rate is defined as the rate of Ca^{2+} uptake during the first 2 s after the dilution of the vesicles in the KCl-medium. The values were corrected for non-specific binding by subtraction of the Ca^{2+} associated to the vesicles diluted in the Na-medium

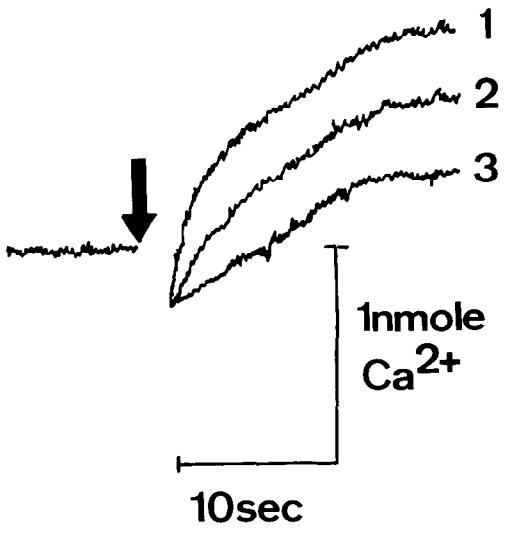

Fig.1. Initial rate of Ca^{2+} uptake on the Na^+/Ca^{2+} exchanger. Technical details in the Materials and methods section and in Caroni et al. [8]. Free Ca^{2+}, 0.5 μM . 5μl of vesicles in Na-medium (50 μg of protein) diluted at arrow in 1 ml of 160 mM KCl, 20 mM Hepes, 1 μM valinomycin, 50 μM Arsenazo III, pH 7.4, 37°C. 1:control; 2:10 μM doxorubicin added to the reaction medium; 3:50 μM doxorubicin added to the reaction medium.

it, or a labile association. The experiment described in table 2 indicates that the association must be rather tight, since the inhibition was still clearly evident, albeit reduced, in sarcolemmal preparations that had been pre-incubated with doxorubicin, and then diluted 200 times in a medium not containing it.

As mentioned, one of us [7] has recently reported that doxorubicin reduces the exchangeability of Ca^{2+}

Table 2
The sodium calcium exchange in heart sarcolemmal vesicles preincubated with doxorubicin

Preincubation with	Initial rate (nmoles Ca^{2+} per mg protein per s)
No addition	9.8
Doxorubicin	
10 μM	6.9
20 μM	5.9
40 μM	5.4

Preincubation of the vesicles for 10 min at 37°C with the concentrations of doxorubicin indicated, then dilution 200 × in a medium containing no doxorubicin and 0.5 μM free Ca^{2+}. Other experimental conditions as in [8] and in table 1

in isolated atria. In principle, the observation could be due to effects of doxorubicin *not* related to influences on the Ca^{2+} transporting membrane systems. One possibility is the direct influence of doxorubicin on the Ca^{2+} non-specifically associated with the sarcolemmal membrane [13]. To test it, sarcolemmal vesicles were exposed to 0.5 μM $^{45}CaCl_2$ in the presence or in the absence of doxorubicin. Energy sources (ATP, Na^+ gradient) were not provided, so that only the external side of the sarcolemmal membrane could have become labeled. As control, 0.5 mM La^{3+}, which is known to displace Ca^{2+} from non specific binding sites in membranes, was used. The amount of $^{45}Ca^{2+}$ bound to the membranes was not altered by doxorubicin, but was markedly decreased, as expected, by La^{3+}. A second alternative possibility would be the decrease of the free Ca^{2+} concentration by doxorubicin. Gosalves et al [14] have claimed that the effect of doxorubicin on heart might be related to its Ca^{2+} chelating properties. However, Gosalves et al. [14] have employed mM concentration of Ca^{2+}, whereas in the present experimental conditions (0.5 μM Ca^{2+} free) doxorubicin failed to influence the free Ca^{2+} concentration in a concentration range where it inhibits the Na^+/Ca^{2+} exchange up to 80%. The decreased exchangeability of Ca^{2+} induced by doxorubicin must therefore be explained differently, and it appears permissible to relate it to the effects on the Na^+/Ca^{2+} exchanger reported in the present paper. Indeed, the transmembrane exchange of Ca^{2+} is the result of the combined functioning of the influx and efflux processes. It requires the entry of Ca^{2+} through the slow Ca^{2+} channel (and, possibly, through the Na^+/Ca^{2+} exchanger) and its ejection via the specific pumping ATPase and through the Na^+/Ca^{2+} exchanger. The latter system, therefore, represents an essential component of the 'cycling' of Ca^{2+} across the sarcolemmal membrane, and it is evident that its inhibition will lead to a decreased 'exchangeability' of Ca^{2+} between heart sarcoplasm and medium.

Whether doxorubicin, in exerting its inhibition, interacts directly with the Na^+/Ca^{2+} exchanger is an open question. The above mentioned persistence of measurable inhibition in membranes diluted after doxorubicin preincubation in solutions essentially free of the inhibitor indicates tight binding, but offers no clues as to the nature of the site of interaction. The recent observation by Goormaghtigh et al. [15] that the inhibitor interacts specifically and with high affinity with acidic phospholipids, as well as earlier indications for the interaction of doxorubicin with phospholipids [13,16], may be relevant to this point. Although doxorubicin under the experimental conditions of the present study does not displace the Ca^{2+} bound to sarcolemma, its interaction with acidic phospholipids in the environment surrounding the exchanger may possibly hinder its function.

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