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SHORT COMMUNICATION

Inhibition by cyclosporin A and butylated hydroxytoluene of the inner mitochondrial membrane permeability transition induced by Adriamycin aglycones

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The anthracycline antibiotic Adriamycin® (AdM*; doxorubicin) exerts considerable oncolytic activity against a variety of leukemias and solid tumors [1, 2]. Clinical use of the drug, however, is limited by severe cardiotoxicity [3]. The biochemical mechanisms underlying this cardiotoxicity have yet to be defined unequivocally [see, for example, Ref. 4].

The 7-deoxy aglycone is a major metabolite of AdM in rat heart [5], in aerobic rat hepatocytes [6], and in some humans [7], and a correlation between aglycone levels and cardiotoxicity has been suggested [8, 9]. Using isolated rat heart mitochondria, we have found that low concentrations (5–20 μM) of the aglycone derivatives of AdM trigger a Ca^{2+}-dependent increase in the permeability of the mitochondrial inner membrane to small (<1500 daltons) solutes [10], modify mitochondrial sulfhydryl groups [11] and oxidize mitochondrial pyridine nucleotides.† We are interested in the possibility that aglycone-induced disruption of mitochondrial function underlies the cardiotoxicity of the parent drug.

Potentially the most serious effect of AdM aglycones on isolated cardiac mitochondria is the loss of the inner membrane as a permeability barrier. Several compounds have been reported to block this permeability transition when it is elicited by a variety of other triggering agents. Cyclosporin A (CsA) inhibits the Ca^{2+}-dependent permeability transition of liver mitochondria, with 50% effectiveness being achieved at 5–50 pmol CsA/mg protein [12, 13]; heart mitochondria are similarly sensitive [14]. It has been proposed that CsA prevents the opening of a membrane pore which mediates the transition process [13, 14]. Butylated hydroxytoluene (BHT; 5–50 μM) slows the Ca^{2+}-dependent permeability transition of liver mitochondria [15–17]. This communication reports the effects of CsA and BHT on the AdM aglycone-induced permeability transition of heart and liver mitochondria.

Methods

Mitochondria were isolated from the hearts of male Sprague–Dawley rats by a procedure [10] which yields a mixed population of interfibrillar and subsarcolemmal organelles. Liver mitochondria were isolated according to Johnson and Lardy [18], but using the same buffers as for the heart preparation. Experiments with heart mitochondria were carried out in 2.5 mL of a standard resin (Chelex-100)-treated buffer, which consisted of 100 mM sucrose, 50 mM KCl, 20 mM MOPS-KOH (pH 7.2), and 1.7 mM KH_2PO_4, to which was added 0.8 μM rotenone and mitochondrial protein equivalent to 0.2 mg/mL. For measurements with liver mitochondria, the phosphate content of the buffer was reduced to 0.2 mM and the mitochondrial protein concentration was increased to 0.4 mg/mL. Ca^{2+} uptake and retention, energized by 5 mM succinate, were monitored continuously with a Ca^{2+}-selective electrode, at Ca^{2+} loads corresponding to 30% of mitochondrial capacity, as previously described [10]. Temperature was maintained at 30°. All data reported are representative of multiple (≥3) experiments.

The 7-hydroxy aglycone of Adriamycin, referred to throughout as AdM aglycone, was prepared as previously described [10] and used in all experiments in place of the physiological metabolite, 7-deoxy AdM aglycone. The effects of the two molecules on heart mitochondria are demonstrably similar† [10, 11], but the former is substantially easier to handle. Adriamycin hydrochloride was supplied by Adria Laboratories, Columbus, OH; cyclosporine (OL 27-400) was the gift of Sandoz Research Institute, East Hanover, NJ. All other reagents were of the highest quality available. Both CsA and BHT were dissolved in 95% ethanol. Solutions of BHT were prepared fresh on the day of the experiment.

Results and Discussion

The major objective of this project was to assess the ability of CsA and BHT to prevent the *AdM aglycone-induced* permeability transition which has been observed with *heart* mitochondria [10]. To permit comparison with earlier studies of BHT, an important first step in the investigation was determination of the effects of AdM aglycone on the permeability transition of isolated *liver* mitochondria. The Ca^{2+}-dependent mitochondrial inner membrane permeability transition was monitored via Ca^{2+} release. As shown in Fig. 1, AdM aglycone (2–60 μM) induced Ca^{2+} release from isolated rat liver mitochondria. The effect was concentration dependent, and liver mitochondria were at least as sensitive to the drug as are heart mitochondria [10].

The capacity of liver mitochondria to accumulate Ca^{2+} is limited relative to that of heart mitochondria. For that reason, the concentration of liver mitochondrial protein used in comparative measurements was set at twice the concentration of heart mitochondrial protein. The aglycone concentration was likewise doubled in experiments with liver mitochondria to maintain a constant ratio of aglycone to mitochondrial protein.

CsA blocked Ca^{2+} release triggered by AdM aglycone both from heart (Fig. 2A) and from liver (Fig. 2B) mitochondria. As has been reported in studies using other agents capable of inducing the permeability transition [13, 14], CsA was effective at concentrations between 0.01 and 0.1 μM. This value falls within the range of binding constants reported for the CsA-binding protein cyclophilin [19], which has been isolated recently from *Neurospora* mitochondria [20], and corresponds to 25–500 pmol/mg mitochondrial protein.

* Abbreviations: AdM, Adriamycin; BHT, butylated hydroxytoluene; CsA, cyclosporine A; DMSO, dimethyl sulfoxide; and MOPS, 3-(*N*-morpholino)propane-sulfonic acid.

† Sokolove PM, manuscript submitted for publication.

In contrast to CsA, BHT (16 μM) delayed aglycone-induced Ca^{2+} release only from liver mitochondria (Fig. 3B). The failure of BHT to reverse the effects of AdM aglycone on heart mitochondria (Fig. 3A) cannot be attributed to a generalized persistence of aglycone effects in this particular heart preparation. In the same experiment, Ca^{2+} retention by heart mitochondria exposed to AdM aglycone in the presence of CsA (0.1 μM) exceeded that observed in the control (data not shown). Similarly, differences in the BHT/aglycone ratio cannot account for differences between mitchondria from the two tissues since that ratio was higher for the heart experiment. The effects

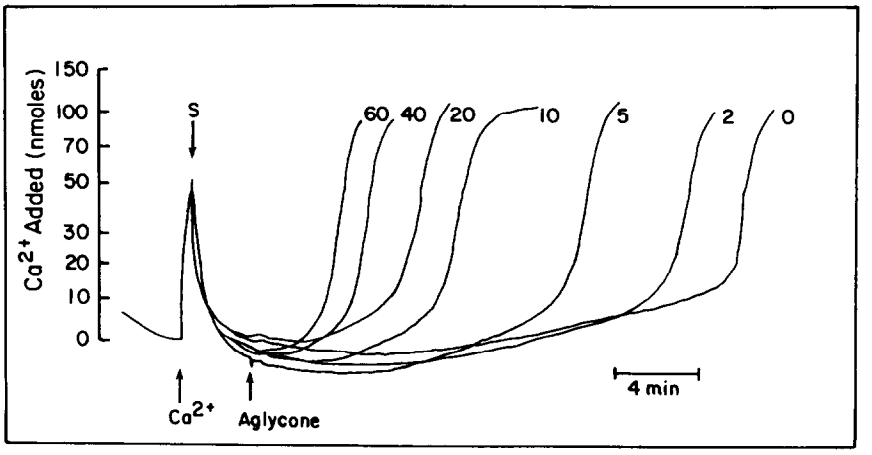

Fig. 1. Effect of AdM aglycone on Ca^{2+} retention by isolated rat liver mitochondria. Mitochondria were preincubated for 3 min in the presence of rotenone. At the arrows, Ca^{2+} (44 nmol), succinate (S; 5 mM), and either AdM aglycone at the concentration (μM) indicated adjacent to the trace or, in the control, dimethyl sulfoxide (DMSO) were added.

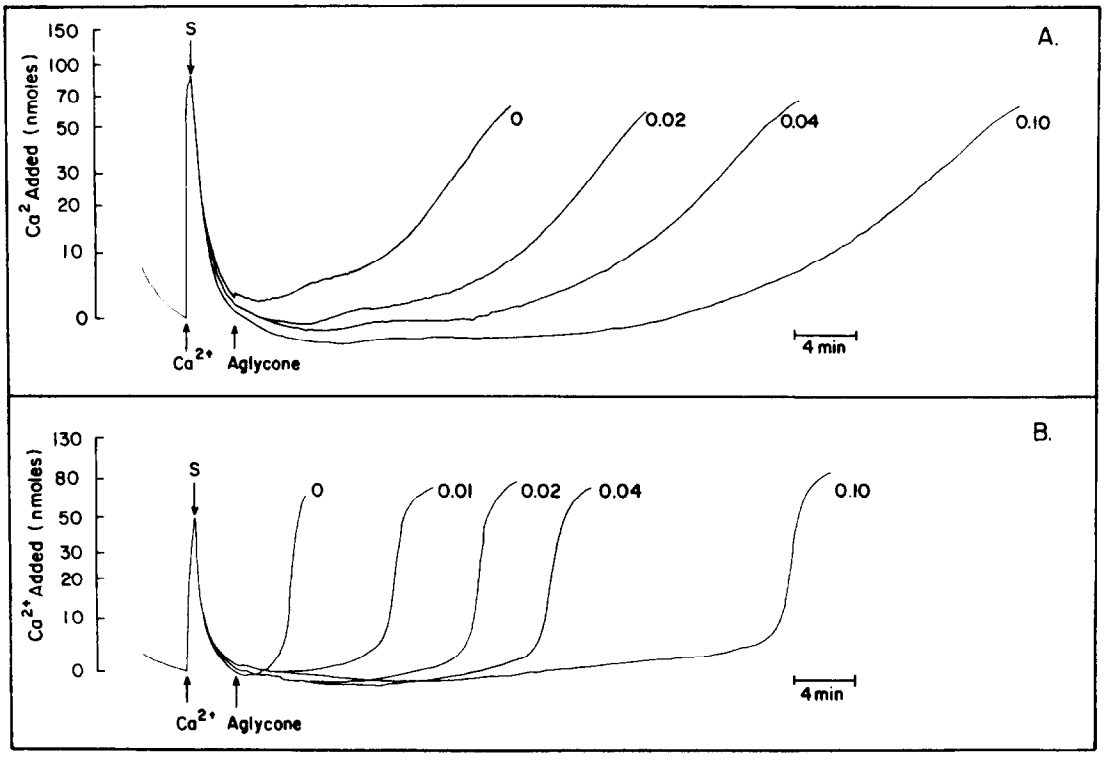

Fig. 2. Effect of CsA on the aglycone-induced release of Ca^{2+} from isolated heart (A) and liver (B) mitochondria. CsA, at the concentrations (μM) shown adjacent to the traces, was present at the outset. Ca^{2+}, succinate (S, 5 mM), and AdM aglycone were added at the points indicated. Panel A: Ca^{2+} added, 84 nmol; aglycone concentration, 20 μM. Panel B: Ca^{2+} added, 74 nmol; aglycone concentration, 40 μM.

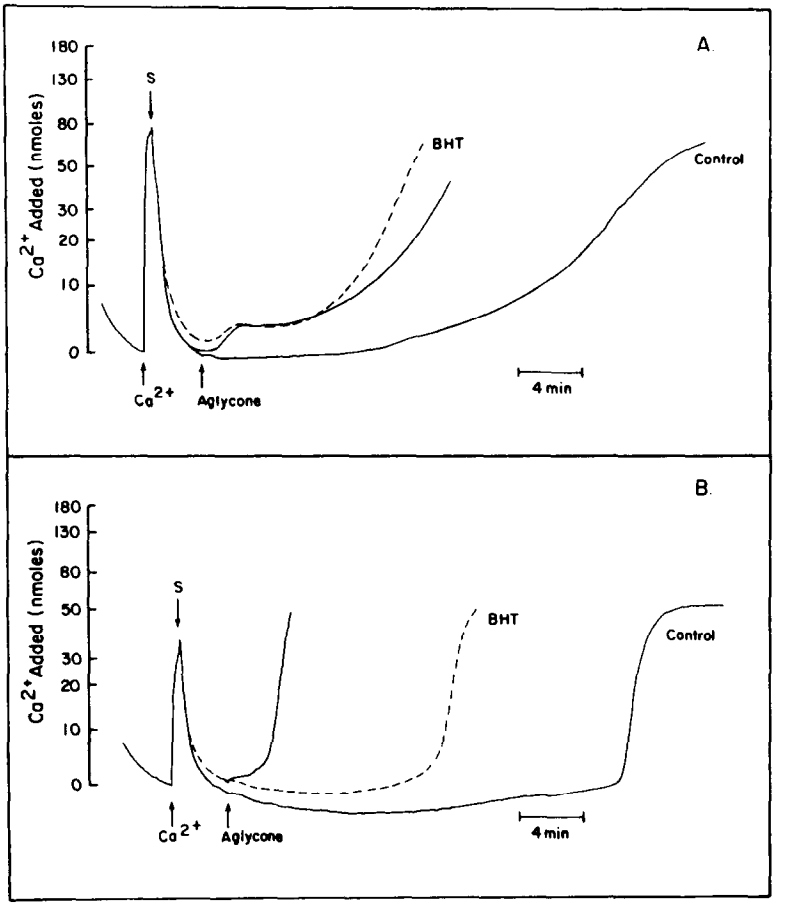

Fig. 3. Effect of BHT on the aglycone-induced release of Ca^{2+} from isolated heart (A) and liver (B) mitochondria. BHT (16 μM; broken lines) was present from the outset. Ca^{2+}, succinate (S, 5 mM) and AdM aglycone were added where indicated. Panel A: Ca^{2+} added, 70 nmol; aglycone concentration, 20 μM. Panel B: Ca^{2+} added, 74 nmol; aglycone concentration, 40 μM. In the controls, DMSO replaced the aglycone.

of BHT are reported to depend on the conditions of mitochondrial incubation, e.g. in the absence of inorganic phosphate BHT *induces* rather than inhibits Ca^{2+} release from liver mitochondria [16]. Therefore, efforts were made to find experimental conditions under which BHT might block the aglycone-induced release of Ca^{2+} from isolated heart mitochondria. BHT effects were measured in both the presence and absence of 1.7 mM inorganic phosphate and at BHT concentrations from 4 to 50 μM. In all cases, BHT effects were minimal (data not shown).

The data summarized above indicate that AdM aglycone induces a Ca^{2+}-dependent permeability transition in mitochondria from liver as well as from heart and that mitochondria from the two tissues are similarly sensitive to the drug. Of key importance, the permeability transition in mitochondria from both tissues was blocked by exceedingly low CsA concentrations (0.01 to 0.1 μM). AdM cardiotoxicity has been reported by several laboratories [8, 9] to be associated with aglycone production. If cardiotoxicity is mediated by the aglycone, CsA may have potential as a cardioprotective agent. Unlike CsA, BHT blocked aglycone-induced Ca^{2+} release only from liver mitochondria. It can be proposed that the mechanisms of interaction of AdM aglycone with heart and liver mitochondria differ.

Note added in proof: Halestrap and Davidson [*Biochem J* **268**: 147–152 and 153–160, 1990] have suggested that CsA blocks the pore responsible for the Ca^{2+}-dependent inner membrane permeability transition by binding to cyclophilin and preventing its interaction with the adenine nucleotide translocase.

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