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Na^+-INDEPENDENT RELEASE OF Ca^{2+} FROM RAT HEART MITOCHONDRIA

INDUCTION BY ADRIAMYCIN AGLYCONE

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Abstract—The effect of adriamycin aglycones on Ca^{2+} retention by isolated, preloaded rat heart mitochondria was assessed. After an initial lag, which decreased with increasing drug concentration, the 7-hydroxy-aglycone (5–20 μM) triggered Ca^{2+} release. Aglycone-induced Ca^{2+} release was correlated with Ca^{2+}-dependent mitochondrial swelling, Ca^{2+}-dependent collapse of the mitochondrial membrane potential, Ca^{2+}-dependent oxidation of mitochondrial pyridine nucleotides, and a transition from the condensed to the orthodox configuration. Aglycone-induced Ca^{2+} release was inhibited by dibucaine, dithiothreitol, ATP, and bovine serum albumin. It can be concluded , therefore, that aglycone-induced Ca^{2+} release reflects the Ca^{2+}-dependent increase in the permeability of the inner mitochondrial membrane to solutes of molecular weight < 1000 which has been observed with other triggering agents [R. A. Haworth and D. R. Hunter, *Archs Biochem. Biophys.* **195**, 460 (1979); I. Al-Nasser and M. Crompton, *Biochem. J.* **239**, 19 (1986)]. In particular, the 7-hydroxy-aglycone decreased the amount of Ca^{2+} required to trigger the permeability increase. No effect of the aglycone on Ca^{2+} uptake could be discerned. 7-Deoxy-adriamycin aglycone, the more prominent biological metabolite of adriamycin, was similarly effective in inducing Ca^{2+} release, and both aglycones were substantially more effective than the parent drug. Adriamycin and related anthracyclines are potent antineoplastic agents, the clinical use of which is limited by severe cardiotoxicity. These results suggest that aglycone formation and the resultant disruption of both cellular Ca^{2+} homeostasis and metabolite compartmentation may mediate anthracycline cardiotoxicity.

Ca^{2+} release from preloaded mitochondria isolated from rat liver or heart can be triggered by Ca^{2+} overload [1, 2] as well as by diverse agents including palmitoyl CoA [3, 4]; high inorganic phosphate (P_i) levels [1, 4]; substrates such as oxaloacetate [5] or *t*-butyl hydroperoxide [6]; sulfhydryl reagents [7]; and toxic substances, e.g. menadione [8], alloxan [9] and divicine [10]. In each of these cases, Ca^{2+} release is associated with mitochondrial swelling, oxidation of mitochondrial pyridine nucleotides, and collapse of the mitochondrial membrane potential ($\Delta\psi$), all of which require prior Ca^{2+} uptake. Transition from the condensed to the orthodox configuration and release of K^+, Mg^{2+} and adenine nucleotides [2, 11] have also been reported to accompany Ca^{2+} release in some instances. The Ca^{2+} release process and the associated events are antagonized by ADP, ATP and Mg^{2+} [12]; by membrane energization [12]; by reduction of mitochondrial pyridine nucleotides [5, 12]; by dibucaine [13]; and by dithioerythritol [14]. Al-Nasser and Crompton [15] have demonstrated recently, by monitoring [^{14}C]sucrose trapping, that triggered Ca^{2+} release is the result of increased mitochondrial permeability to low molecular weight solutes. Permeabilization is reversed rapidly when Ca^{2+} concentration is lowered, permitting restoration of $\Delta\psi$. Triggered Ca^{2+} release, therefore, can be viewed as evidence of reversible, Ca^{2+}-dependent modulation of mitochondrial inner membrane permeability.

We have identified the naphthacenecarboxamide antibiotic chlortetracycline as an additional, and unexpectedly potent, agent capable of triggering Na^+-independent Ca^{2+} release from isolated rat heart mitochondria [16]. This paper demonstrates that adriamycin aglycone, a compound which chemically resembles chlortetracycline (Scheme 1), triggered Ca^{2+} release by the same mechanism at concentrations as low as 5 μM.

Adriamycin (doxorubicin) is a potent antineoplastic agent [17], but its clinical use is limited by cumulative cardiotoxicity [18]. Several hypotheses have been advanced to account for the toxic effects of the drug [for a review see Ref. 19], including (1) inhibition of coenzyme Q_{10}-dependent enzymes [20], (2) reduction of adriamycin to form a free radical [21] with subsequent generation of activated oxygen species [22] and lipid peroxidation [23], (3) alteration of mitochondrial respiration [24] or Ca^{2+} metabolism [25, 26], (4) interaction with the mitochondrial lipid cardiolipin [27] leading to inhibition of phosphate translocation [28] or of electron flow through cytochrome *c* oxidase [29], and (5) membrane destruction mediated by an adriamycin–Fe(III) complex [30]. Among these processes, only interaction of adriamycin with cardiolipin and damage due to drug–Fe(III) complexes have been demonstrated to occur at adriamycin concentrations (<50 μM) that might

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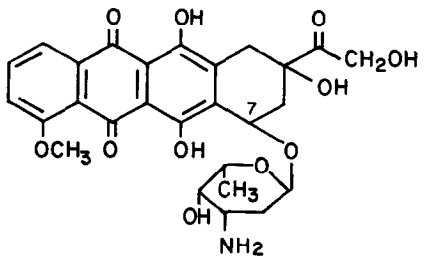
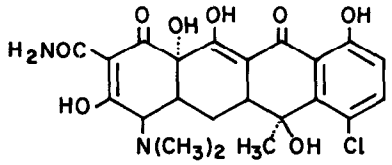


Adriamycin (Doxorubicin)



Chlortetracycline

Scheme 1. Chemical structures of chlortetracycline and adriamycin. In adriamycin aglycone and 7-deoxy-aglycone, the sugar at C-7 is replaced by —OH and —H respectively.

be achieved during therapeutic administration of the drug [31]. None of the processes has been implicated unequivocally in the induction of cardiomyopathies [19].

Adriamycin aglycones are produced under anaerobic conditions by mitochondria and microsomes [32] as the result of reductive cleavage of the parent molecule. The results summarized here demonstrate that the aglycones examined, at 5 μM, altered mitochondrial Ca^{2+} retention and the permeability properties of the inner mitochondrial membrane. (A brief earlier report [26] indicated that 100 μM aglycone stimulates Ca^{2+} release). The following sequence of events can therefore be proposed to mediate the cardiotoxic effects of adriamycin: (1) the parent drug accumulates in mitochondria due to its high affinity for cardiolipin, (2) localized, and possibly transient, O_2 depletion within working heart mitochondria permits formation of the aglycone, and (3) the aglycone decreases the ability of cardiac mitochondria to retain Ca^{2+} and other metabolites with a consequent disruption of cellular homeostasis.

MATERIALS AND METHODS

Isolation of mitochondria. Heart mitochondria were isolated from male Sprague–Dawley rats (350–500 g) as described by Sordahl [33]. Briefly, the animals were anesthetized with CO_2 and decapitated. Hearts were rapidly excised, trimmed to remove atria, and minced. A 5% suspension of tissue in Buffer 1 (250 mM sucrose, 5 mM HEPES*-KOH, pH 7.2, 1 mM EGTA) was disrupted with a Polytron PT-10/35 (setting 7, 3 sec). The resulting brei was centrifuged (27,000 *g*, 10 min) and the pellet incubated with Nagarse (1 mg/g heart tissue), suspended at 1 mg/ml in Buffer 2 (180 mM KCl, 10 mM HEPES-KOH, pH 7.2, 0.5% BSA) supplemented with 1 mM EGTA, to release interfibrillar mitochondria. After 8 min the suspension was made 5% in Buffer 2 plus EGTA and homogenized with a loose-fitting Teflon pestle. Cellular debris was removed by low-speed centrifugation (400 *g*, 5 min). The mitochondria were pelleted at 10,000 *g* (10 min), washed three times in Buffer 2, and resuspended in Buffer 2 (1 ml/g heart tissue). The resultant mixed population of subsarcolemmal and interfibrillar mitochondria generally contained about 12 mg protein/g heart tissue.

Measurement of mitochondrial Ca^{2+} *fluxes.* Ca^{2+} uptake and release were monitored by means of a Ca^{2+}-selective electrode (Radiometer, F2112Ca, K8040 reference) in a standard, resin (Chelex-100)-treated reaction buffer (2 ml) consisting of sucrose, 100 mM; KCl, 50 mM; MOPS-KOH, pH 7.2, 20 mM; KH_2PO_4, 1.7 mM; to which were added 0.8 μM rotenone; and 0.4 mg mitochondrial protein. Na^+ salts were avoided in all cases to preclude operation of the mitochondrial Na^+/Ca^{2+} exchanger. The mitochondria were preincubated with rotenone for 3 min prior to initiation of experimentation and energized with 1.7 or 5 mM succinate; temperature was maintained at 30°. All measurements were conducted in siliconized beakers. Alternatively, Ca^{2+} uptake and release were followed under the same conditions in a siliconized cuvette in the presence of Arsenazo III (30 μM) in an SLM-Aminco DW-2C dual wavelength spectrophotometer at the wavelength pair 675–685 nm [34]. Of the Ca^{2+} data presented in this publication only Fig. 7 was obtained spectrophotometrically.

The ability of mitochondrial preparations to accumulate Ca^{2+} is variable. Preparations were therefore standardized as follows. After energization, Ca^{2+} (50 nmol) was added and uptake was monitored until a constant extramitochondrial free Ca^{2+} concentration was reached. Sequential similar additions of Ca^{2+} were made until the onset of spontaneous Ca^{2+} release from the mitochondria. The total Ca^{2+} accumulation at the point of release was defined as the uptake capacity of the preparation, a value ranging from 600 to 1000 nmol/mg protein under standard conditions. Ca^{2+} additions during experiments were normalized to this value. Electrode or metallochromic indicator response was calibrated by addition of known amounts of Ca^{2+}.

Other assays. Oxygen consumption was measured polarographically under Ca^{2+}-uptake conditions with a Clark-type oxygen electrode [35]. ADP (600 nmol) was added to permit the calculation of respiratory control ratio (RCR). Respiratory control ratios in this low (1.7 mM) phosphate buffer were routinely 5–8 with succinate and rotenone, increasing to > 12 when measured with glutamate and malate (8 mM, each) in the presence of 10 mM inorganic phosphate. Under the experimental conditions employed (30°, low mitochondrial protein concentration, rapid stirring), samples did not become anaerobic, even in the longest incubations reported. Mitochondrial swelling was monitored as a decrease in apparent absorbance

* Abbreviations: BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; EGTA, ethyleneglycolbis(aminoethyl-ether)tetra-acetate; F-CCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; HEPES, *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid; and MOPS, *N*-morpholino-propanesulfonic acid.

at 540 nM. Oxidation of pyridine nucleotides was followed at 340–370 nm in an Aminco DW-2C dual wavelength spectrophotometer. Membrane potential was measured qualitatively at 533–511 nm using the potential-sensitive dye safranine [36]. Because the dye itself tended to promote Ca^{2+} release, it was present at a concentration of only 1.5 μM. All data shown are from representative experiments. Protein was determined according to Lowry *et al.* [37] with BSA as standard.

Electron microscopy. Samples (0.5 ml) for electron microscopy were removed at various points of the Ca^{2+} uptake and release cycle and mixed rapidly with 50 μl of 50% glutaraldehyde. After incubation for 5 min at 4°, the mitochondria were pelleted (15,000 *g*, 5 min). Pellets were rinsed with buffer, postfixed in 1% OsO_4 containing 1.5% $K_3Fe(CN)_6$, stained in block with 5% aqueous uranyl acetate, dehydrated through an ethanol series, and embedded in an EM-bed 812/araldite mixture. Ultrathin sections were stained with methanolic uranyl acetate followed by lead citrate and examined with a Siemens Elmiskop 101 electron microscope at 80 kV.

Preparation of adriamycin aglycones. 7-Hydroxy-aglycone (referred to as adriamycin aglycone) was prepared by heating the parent compound at 100° for 30 min in the presence of 0.1 N HCl and extracting the cleavage product into toluene [38]. The 7-deoxy-aglycone was prepared as described by Smith *et al.* [39], utilizing reductive cleavage of adriamycin (in tetrahydrofuran/methanol) by sodium dithionite and extraction of the hydrophobic product into methylene chloride. All glassware utilized for these procedures was siliconized. Aglycones were analyzed by TLC on silica gel G plates developed in ethyl acetate/ethanol/glacial acetic acid/water (80:10:5:3). Plates were pre-run in the same solvent prior to use. In agreement with previous reports [40], the R_f values for the 7-hydroxy- and 7-deoxy-aglycones were 0.72 and 0.84 respectively. Neither algycone preparation contained detectable amounts of the parent compound or of the other aglycone. Aglycones were dissolved in DMSO and quantitated spectrophotometrically by reference to the parent compound in 0.6 N HCl in 95% ethanol [31] using an extinction coefficient for adriamycin at 495 nm of

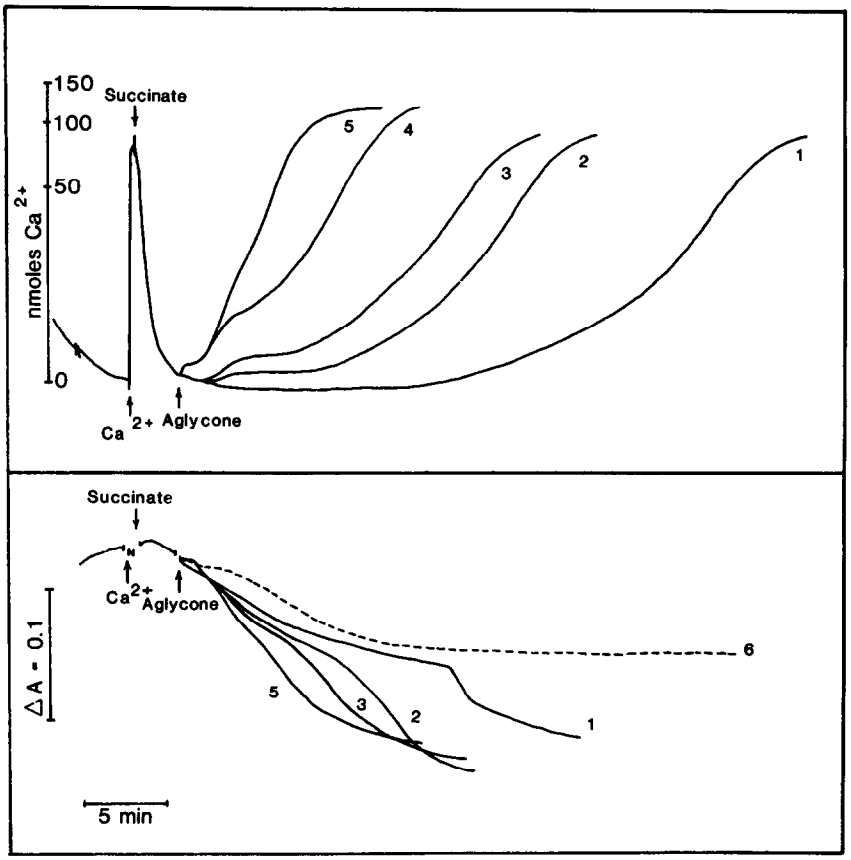


Fig. 1. Effect of adriamycin aglycone on Ca^{2+} retention by isolated rat heart mitochondria and on mitochondrial swelling. Upper panel: ionized Ca^{2+} concentration determined with a Ca^{2+}-selective electrode. Electrode response to standard additions of Ca^{2+} is indicated on the left. Lower panel: absorbance at 540 nm. Ca^{2+} (64 nmol, 30% loading), succinate (1.7 mM), and aglycone (or DMSO) were added at the points indicated. Trace 1: control, 4 μl DMSO added in place of aglycone. This corresponds to the largest solvent volume used (traces 5 and 6). Trace 2: 5.1 μM aglycone. Trace 3: 10.2 μM aglycone. Trace 4: 15.3 μM aglycone. Trace 5: 20.4 μM aglycone. Trace 6: 20.4 μM aglycone added in the absence of Ca^{2+}. All measurements were made in parallel with a single mitochondrial preparation. Other details are outlined under Materials and Methods.

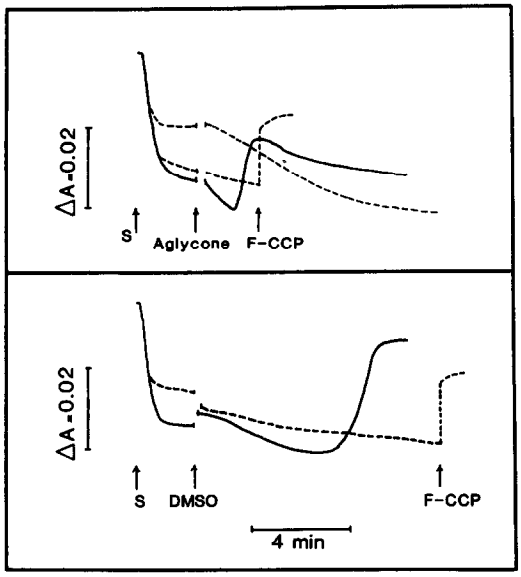


Fig. 2. Effect of adriamycin aglycone on mitochondrial membrane potential. Mitochondria supplemented with 1.5 μM safranine were subjected to the standard protocol shown in Fig. 1 either in the presence (solid lines) or absence (broken lines) of Ca^{2+} (49 nmol, 25% loading). Addition of succinate (5 mM), and 2.5 min later aglycone (15 μM, upper panel) or an equivalent volume of DMSO (1.7 μl, lower panel) is indicated by the arrows. F-CCP (1.25 μM) was added to runs without Ca^{2+} where indicated. Other details were as described in Materials and Methods.

12,934 $M^{-1} \cdot cm^{-1}$ [41]. Experimentation focused on the 7-hydroxy-aglycone. This molecule was produced in higher yield with fewer minor contaminants than the 7-deoxy-aglycone. Because it is less hydrophobic, effects of the 7-hydroxy form could be monitored via a Ca^{2+}-selective electrode.

Materials. Adriamycin hydrochloride was supplied by Adria Laboratories (Columbus, OH). Sucrose, HEPES, EGTA, MOPS, BSA, ADP, rotenone, Nagarse, Sigamacote, and, in some instances, adriamycin were purchased from the Sigma Chemical Co. (St. Louis, MO). Safranine-O and F-CCP were from Aldrich (Milwaukee, WI), and Chelex-100 from BioRad (Richmond, CA). All other reagents were of the highest quality available.

RESULTS

Effect of 7-hydroxy adriamycin aglycone (adriamycin aglycone) on mitochondrial Ca^{2+} *retention.* Adriamycin aglycone at low concentrations (≤20 μM) induced the release of Ca^{2+} from preloaded rat heart mitochondria (Fig. 1, top panel). Release was biphasic and occurred with a lag which was shortened as the aglycone concentration was increased. The amount of Ca^{2+} released was independent of the amount of aglycone added; regardless of aglycone concentration, all accumulated Ca^{2+} was released. Ca^{2+} was also released from preloaded rat heart mitochondria, albeit more slowly, in the absence of adriamycin aglycone (trace 1).

Aglycone-induced Ca^{2+} release was accompanied by mitochondrial swelling as indicated by a decrease in apparent absorbance at 540 nm (Fig. 1, lower panel). The onset of swelling was dependent on aglycone concentration and was correlated in time with the onset of Ca^{2+} release. Aglycone-induced swelling was largely dependent on the presence of Ca^{2+}. Traces 5 and 6 were recorded in the presence of 20 μM aglycone. They differ in that Ca^{2+} was omitted from 6.

Aglycone-induced Ca^{2+} release was also associated with collapse of the mitochondrial membrane potential, indicated by an increase in safranine absorbance (533−511 nm) (Fig. 2). The lag preceding membrane potential collapse was greatly reduced by adriamycin aglycone. As observed for swelling, collapse of $\Delta\psi$, whether aglycone-induced or spontaneous, was dependent on the presence of Ca^{2+} (Fig. 2). The uncoupler F-CCP (1.25 μM) induced an increase in the safranine signal of similar magnitude which was Ca^{2+} independent. Addition of F-CCP after the aglycone-induced increase in the safranine signal had no effect (not shown), indicating that the spectral changes elicited by the aglycone represented authentic membrane depolarization. Membrane potential collapse occurred more rapidly

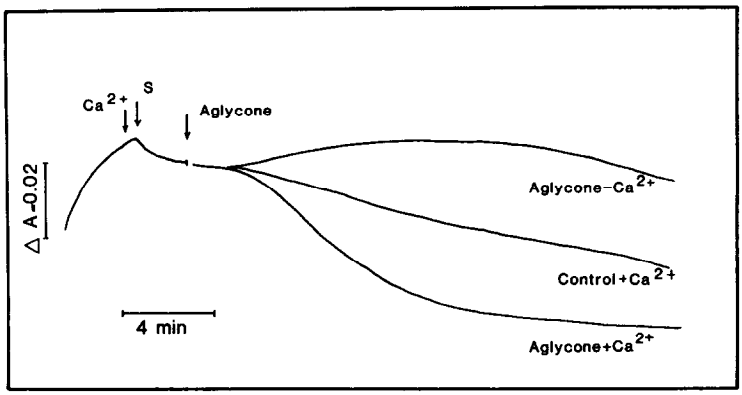


Fig. 3. Effect of adriamycin aglycone on the redox state of mitochondrial pyridine nucleotides. Ca^{2+} (46 nmol, 20% loading), succinate (5 mM) and aglycone (15 μM) or an equivalent volume of DMSO (1.7 μl) were added as indicated. Other details are given in Materials and Methods. Pyridine nucleotide oxidation is indicated by a decrease in signal.

than other aglycone-induced processes (compare Figs. 1–3). This can be attributed to an effect of the dye on Ca^{2+} retention. When safranine was added to the reaction mixture, Ca^{2+} release, measured either in the presence or absence of aglycone, was correspondingly speeded (data not shown).

As outlined above, aglycone-induced Ca^{2+} release was correlated with mitochondrial swelling and $\Delta\psi$ collapse. The process was also associated with Ca^{2+}-dependent oxidation of mitochondrial pyridine nucleotides (Fig. 3) and transition from the condensed to the orthodox configuration (Fig. 4). This constellation of associated phenomena can be considered diagnostic for the Na^+-independent, pyridine nucleotide-linked Ca^{2+} release process [4, 11] which reflects altered mitochondrial membrane permeability [15].

Inhibition of aglycone-induced Ca^{2+} *release.* Pfeiffer and coworkers [13] have characterized triggered Ca^{2+} release as being inhibited by dibucaine. In heart mitochondria, dibucaine effects were dependent on the ratio of anesthetic to mitochondrial protein and on Ca^{2+} load. At ratios higher than 750 nmol dibucaine/mg protein or at Ca^{2+} loads > 20%, dibucaine induced Ca^{2+} release. Inhibition of aglycone-induced release, therefore, could be demonstrated only under carefully selected conditions and was, in any case, minimal (data not shown).

Two other agents reported to block triggered Ca^{2+} release have, however, been identified as effective inhibitors of aglycone-induced release. Both dithiothreitol (2 mM) and ATP (50 μM) slowed aglycone-induced Ca^{2+} release from preloaded rat heart mitochondria (Fig. 5). ATP chelates Ca^{2+}. It can be calculated, using the programs of Fabiato and Fabiato [42], that for the experiment in Fig. 5, in the presence of 50 μM ATP, addition of 90 nmol of Ca^{2+} to the 2-ml reaction mixture produced an ionized Ca^{2+} concentration of only 37.3 μM. In subsequent experiments, adjustment of Ca^{2+} additions such that the same ionized Ca^{2+} concentrations were achieved in the presence and absence of ATP produced results indistinguishable from those in Fig. 5 (data not shown). Inhibition of Ca^{2+} release by ATP, therefore, must be attributed to a factor other than Ca^{2+} chelation. Sulfhydryl reagents have been proposed to block Ca^{2+} release by maintaining crucial protein sulfhydryl groups reduced [14]; adenine nucleotides are thought to act by complexing the adenine nucleotide translocator [12]. Whatever the mechanisms involved, the inhibitory effects of both dithiothreitol and ATP were exerted on both aglycone-induced and spontaneous Ca^{2+} release.

Mitochondria utilized in these experiments were resuspended in Buffer 2 which contained 0.5% BSA. They were generally added to the standard (2.0 ml) assay mixture in a volume of 25–30 μl, producing a final BSA concentration of 0.006 to 0.008%. An increase of BSA concentration to 0.025% slowed aglycone-induced Ca^{2+} release (data not shown). Although BSA has been identified previously as an inhibitor of triggered Ca^{2+} release [43], in this system, direct inhibition cannot be distinguished from a decrease in effective aglycone concentration due to complexation by BSA.

Mechanism of aglycone-induced Ca^{2+} *release.* Under the conditions of these experiments, effects of adriamycin aglycone on mitochondrial respiration were minor. In the absence of Ca^{2+}, state 2 and state 4 rates of oxygen consumption and ADP/O values [35] were unchanged (data not shown). The highest drug concentration utilized (20 μM) decreased state 3 (+ADP) respiration minimally (21.6 ± 2.9%; three experiments). Aglycone-induced Ca^{2+} release, therefore, cannot be attributed to either inhibition of electron flow or uncoupling.

The dependence on Ca^{2+} concentration of aglycone effects on Ca^{2+} uptake and release was determined (Fig. 6). Aglycone (broken lines) or an equivalent volume of DMSO (solid traces) was added to mitochondria 3 min prior to Ca^{2+}. Panels A through F represent the results obtained for Ca^{2+}

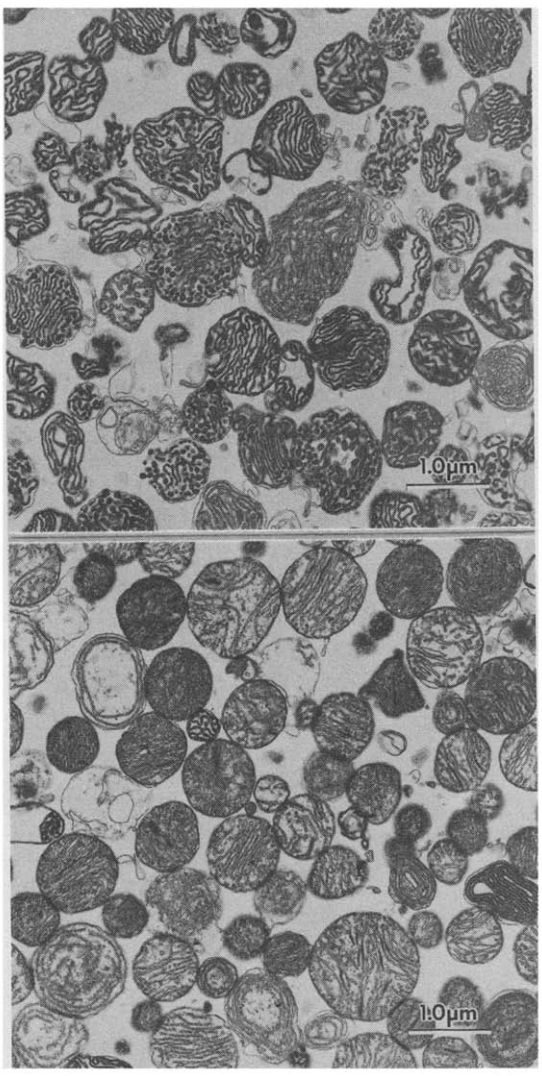


Fig. 4. Effect of adriamycin aglycone on mitochondrial configuration. Mitochondria were treated as for trace 4, Fig. 1. Upper panel: mitochondria fixed after 3-min preincubation in standard buffer supplemented with rotenone and prior to the addition of 55 nmol Ca^{2+} (25% loading). Lower panel: mitochondria fixed after the completion of Ca^{2+} release induced by 15 μM adriamycin aglycone. Other details are outlined in Materials and Methods.

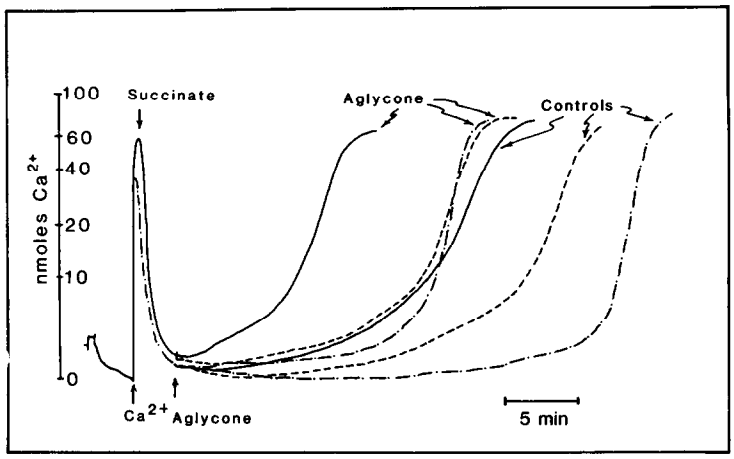


Fig. 5. Effect of dithiothreitol and ATP on Ca^{2+} release from rat heart mitochondria. Mitochondria were preincubated for 3 min in the presence of rotenone and either no further addition (———), 50 μM ATP (–·–·–), or 2 mM dithiothreitol (– – –). At the points indicated, additions of Ca^{2+} (90 nmol, 30% loading), succinate (1.7 mM) and either aglycone (10 μM) or an equivalent volume of DMSO (controls) were made.

additions of 26–152 nmol (10–60% loading). Initial uptake rates in the presence and absence of aglycone were, to the extent that can be determined with this methodology, identical.

The data of Fig. 6 suggest that adriamycin aglycone decreased the amount of Ca^{2+} required to trigger the Ca^{2+} release process. In the absence of aglycone, an increase of Ca^{2+} load from 10 to 30% (A–C) had little effect on the time required for complete Ca^{2+} release. In contrast, the lag preceding complete release in the presence of 15 μM aglycone was shortened markedly. At high Ca^{2+} loads (E and F), Ca^{2+} uptake was incomplete, and very little effect of the aglycone on the subsequent rapid release could be observed.

Comparison of the effects of adriamycin aglycone and 7-deoxy-aglycone on Ca^{2+} *retention by isolated rat heart mitochondria.* Metabolic cleavage of adriamycin yields at least a dozen distinguishable aglycones [44]. The 7-deoxy-aglycone is generally regarded as the major product, with 7-hydroxy-aglycone somewhat less important [44, 45]. The 7-deoxy-aglycone, therefore, was prepared and compared, in terms of its effects on mitochondrial Ca^{2+} retention, with the 7-hydroxy derivative and the parent compound (Fig. 7). Traces obtained with two mitochondrial preparations are included to indicate the degree of variability inherent in these measurements. In each case, the 7-deoxy- and 7-hydroxy-aglycones were equivalently effective in inducing Ca^{2+} release. Adriamycin was significantly less potent. Differences in apparent potency may, of course, reflect differences in drug concentration in the inner mitochondrial membrane and/or differences in mechanism of action between the parent drug and its uncharged metabolites.

DISCUSSION

Adriamycin aglycone (5–20 μM) induced the release of Ca^{2+} from isolated, preloaded rat heart mitochondria. Ca^{2+} release was associated with Ca^{2+}-dependent mitochondrial swelling, pyridine nucleotide oxidation, and membrane potential collapse as well as with a configurational transition. The cluster of phenomena associated with Ca^{2+} release and the inhibitor sensitivity of the process indicate that the aglycone activates the Ca^{2+}-dependent mechanism whereby the permeability of the inner mitochondrial membrane to low molecular weight (<1000 daltons) solutes is modulated [15, 46]. In particular, adriamycin aglycone appears to decrease the concentration of Ca^{2+} required to trigger the process.

Aglycone-induced Ca^{2+} release is clearly distinct from the second major release mechanism of cardiac mitochondria, namely Na^+/Ca^{2+} exchange [47]. Measurements were conducted in the absence of added Na^+. The concentration of Na^+ required to elicit half the maximal exchange rate is 8 mM [47]. Trace amounts of Na^+ in reagents would therefore be unable to support significant rates of exchange. Furthermore, in contrast to triggered Ca^{2+} release, Na^+/Ca^{2+} exchange is not associated with swelling [43] or collapse of $\Delta\psi$ [48]. Finally, Na^+/Ca^{2+} exchange is not inhibited by ATP [43] or dithiotreitol [49].

Alteration in the permeability properties of a membrane responsible for segregating organelle contents from cytosol would have marked effects on cellular function. It can therefore be proposed that aglycone effects on cardiac mitochondria are involved in adriamycin cardiotoxicity. This would require, first, that aglycones are produced in heart tissue; second, that the aglycones produced are active; and, finally, that conditions within cardiac cells are permissive of Ca^{2+}-dependent modulation of mitochondrial membrane permeability.

Anthracycline aglycones have, in fact, been reported to accumulate in heart tissue. Mhatre and coworkers [50, 51] reported that, in Syrian golden hamsters, as much as 10–15% of a large (25 mg/kg) dose of adriamycin was found as the aglycone 5–

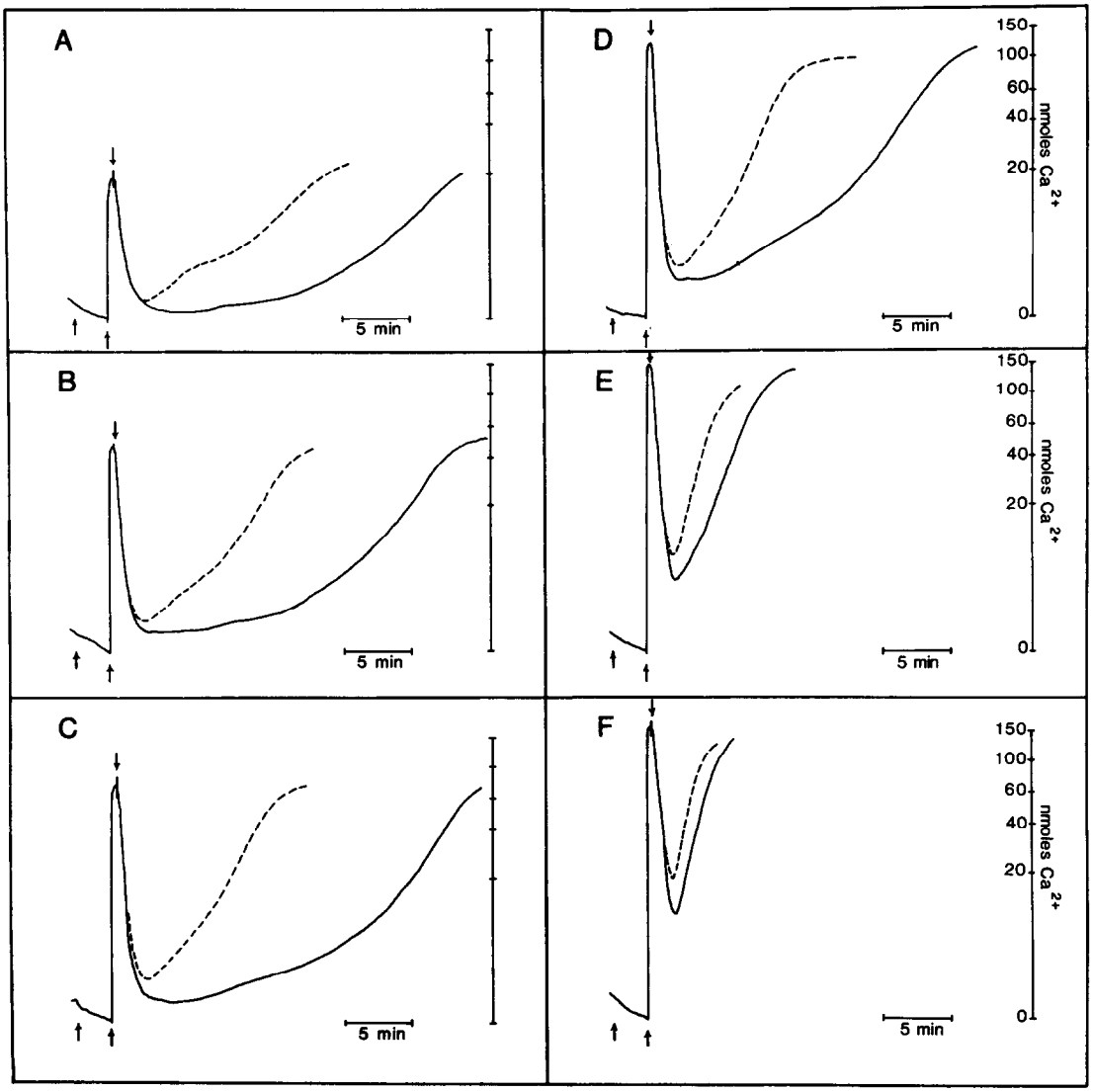


Fig. 6. Dependence of aglycone-induced Ca^{2+} release on mitochondrial Ca^{2+} loading. Aglycone (15.3 μM, broken lines) or an equivalent volume of DMSO (3 μl, solid lines) was added at the point indicated by the first arrow. After a 3-min incubation, Ca^{2+} (second arrow) and succinate (1.7 mM, third arrow) were added. The amounts of Ca^{2+} added, and the resultant percentages of Ca^{2+} loading, were: (A) 26 nmol, 10%; (B) 52 nmol, 20%; (C) 76 nmol, 30%; (D) 102 nmol, 40%; (E) 128 nmol, 50%; and (F) 152 nmol, 60%.

30 min after injection. Aglycone accumulation was particularly great in heart, and, for a series of adriamycin analogs, cardiotoxicity was correlated with aglycone concentration. More recently, Cummings and coworkers [52] have identified adriamycin 7-deoxy-aglycone and adriamycinol-7-deoxy-aglycone as the major metabolites in rat heart 24–48 hr after acute exposure to adriamycin. Similarly, Gewirtz and Yanovich [53] report that the 7-deoxy-aglycone is the primary metabolite when aerobic rat hepatocytes are exposed to adriamycin. Removal of quinone function and thus the potential for aglycone formation has been found to decrease cardiotoxicity [54]. Finally, in an exhaustive study, Peters *et al.* [45] have demonstrated a correlation between cardiotoxicity, the ability of anthracyclines to stimulate O_2 reduction, and efficacy of conversion of the anthracyclines to their aglycones.

In contrast, Bachur and coworkers have found little evidence of aglycone accumulation either at short times after injection in the hamster model [55] or at longer intervals in the rabbit [56]. Additionally, they have reported that, in mice, simultaneous i.p. injection of adriamycin and a radical dimer scavenger decreases cardiotoxicity and increases aglycone levels in the heart [57]. Likewise, Mimnaugh *et al.* found that aglycones were not detectable in mouse heart [58] and very low in rabbit heart [59] 1 hr after adriamycin injection (15 and 5 mg/kg respectively). This controversy has been reviewed recently by Cummings *et al.* [60]. They conclude that aglycones are produced in mice, rats, and, with great inter-patient

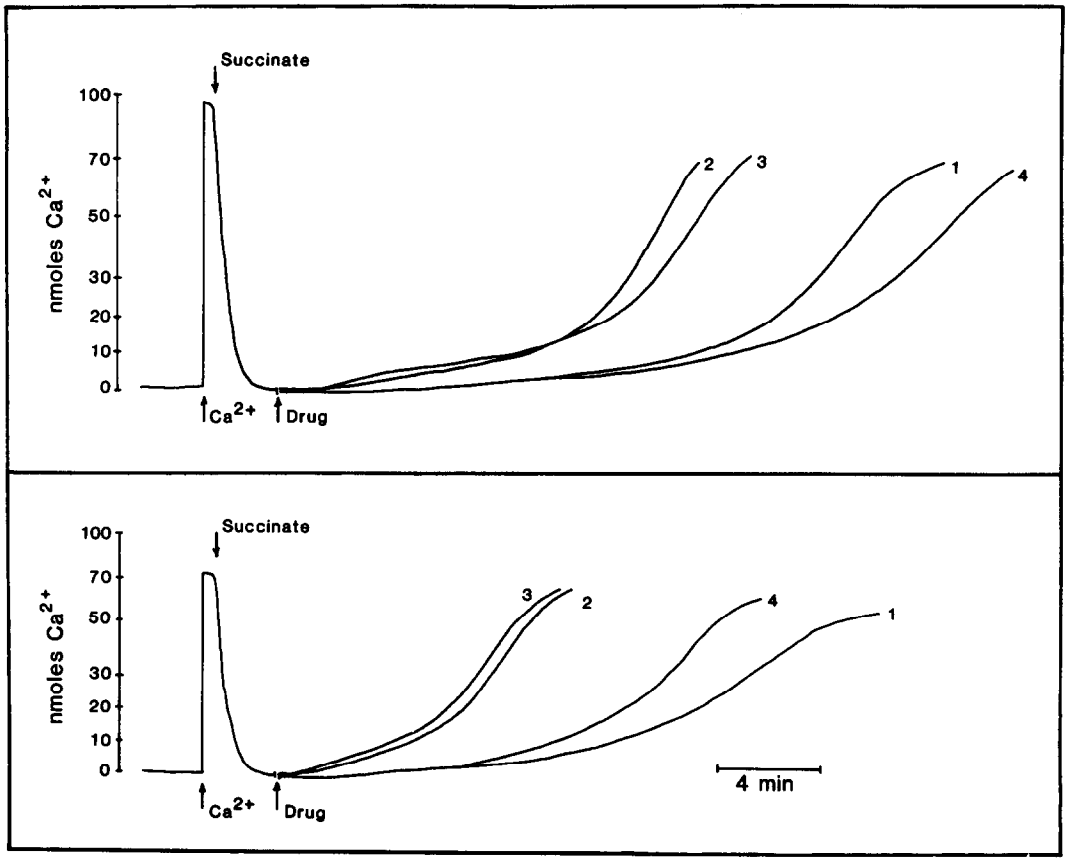


Fig. 7. Comparison of the abilities of adriamycin, adriamycin aglycone, and the 7-deoxy-aglycone to induce Ca^{2+} release from preloaded rat heart mitochondria. Results are shown for two different mitochondrial preparations. In each case, 30% Ca^{2+} loading was employed. This required 81 nmol for the experiment shown in the upper panel and 71 nmol for that presented in the lower. Succinate (5 mM) and drug (or 2.2 μl DMSO) were added as indicated. Additions were: trace 1, DMSO, trace 2, 15 μM aglycone; trace 3, 15 μM 7-deoxy-aglycone; and trace 4, 15 μM adriamycin + DMSO to ensure equivalent solvent composition in all runs.

variation, in humans. The presence of aglycone levels sufficient to effect a Ca^{2+}-dependent alteration in the permeability properties of the inner mitochondrial membrane must be considered possible.

Although at least a dozen different aglycones can be detected upon incubation of anthracyclines with rat liver microsomes [44], there is general agreement that 7-deoxy-aglycones are the major physiological aglycone metabolites [44, 45, 52, 53], with the 7-hydroxy form accumulating to a lesser extent. It is thus of particular interest that this study indicates the 7-deoxy product to be detrimental to mitochondrial Ca^{2+} retention. It is also of interest that in a microsomal system aglycones are further metabolized via C-13 side chain reduction whereas in mitochondria the 7-deoxy-aglycone is the final product [32].

The conditions used in this study to demonstrate the effects of adriamycin aglycones on mitochondrial Ca^{2+} retention are arguably nonphysiological. Of particular concern are the high Ca^{2+} concentrations (13–75 μM, Fig. 6) employed. Several factors argue that the same aglycone effects could be observed *in vivo* at markedly lower Ca^{2+} concentrations. First, rotenone was included in these experiments to eliminate pyridine nucleotide oxidation state as a variable. As previously reported [43], in the absence of rotenone, the Ca^{2+} concentration required to trigger the Ca^{2+} release process was decreased by a factor of 2 to 4 (data not shown). Similarly, an increase in temperature, as shown by Roman *et al.* [61], favors Ca^{2+} release. At 37° the amount of triggering Ca^{2+} required was again decreased 2- to 4-fold relative to 30° measurements (data not shown). Therefore, it can be predicted that aglycone effects would be observed *in vivo* at physiological Ca^{2+} concentrations (0.05 to 5 μM [62]). BSA decreased mitochondrial sensitivity to adriamycin aglycone, suggesting that effects of the drug could also be expressed at lower aglycone concentrations *in vivo*.

The finding that adriamycin aglycones decreased mitochondrial Ca^{2+} retention suggests a novel interpretation for several observations pertinent to anthracycline cardiotoxicity. α-Tocopherol [63] and sulfhydryl-containing reagents [64] have, under some conditions, been reported to reduce anthracycline cardiotoxicity. An involvement of lipid peroxidation

in the toxic event has therefore been inferred. The Ca^{2+}-dependent process which modulates mitochondrial membrane permeability is also sensitive to mitochondrial sulfhydryl status [7, 14]. Perhaps the protective effects of vitamin E and cysteine reflect interference with aglycone effects on mitochondria.

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