Vol. 129, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1985 Pages 18-25

DIVICINE INDUCES CALCIUM RELEASE FROM RAT LIVER MITOCHONDRIA

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Received March 15, 1985

Divicine, a pyrimidine aglycone strongly implicated in the pathogenesis of favism, induces calcium release from intact rat liver mitochondria. Divicine-dependent calcium release is accompanied by oxidation and hydrolysis of intramitochondrial pyridine nucleotides. Inhibition of both mitochondrial glutathione peroxidase and glutathione reductase slows down divicine-induced calcium release. Cyanide-insensitive respiration indicates redox cycling of divicine in mitochondria. The results suggest that attention should be paid to the action of divicine in cells other than red blood cells.

Favism is an acute hemolytic disease affecting glucose-6-phosphate dehydrogenase-deficient subjects. The pyrimidine aglycone divicine has been implicated as one of the components of fava beans that produce damage to red blood cells of such subjects (1-3). In the presence of oxygen, divicine undergoes rapid autoxidation with formation of H_2O_2. Divicine can also oxidize GSH to GSSG nonenzymatically. Furthermore, direct oxidation of pyridine nucleotides by divicine in the presence of glucose-6-phosphate and glucose-6-phosphate dehydrogenase has been reported (4).

To date, divicine has been studied almost exclusively in red blood cells (P. Arese, personal communication). Since the redox

Abbreviations: Arsenazo III, 2,2'-(1,8-dihydroxy-3,6-disulphonaphthalene-2,7-bis azo)-bis-(benzenarsonic acid); BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CCCP, carbonyl-cyanide m-chlorophenylhydrazone; EGTA, ethyleneglycol bis (β-aminoethyl-ether)-N,N,N',N'-tetraacetic acid; $\Delta\Psi$, mitochondrial transmembrane electrical potential, negative inside; MSH, 210 mM mannitol, 70 mM sucrose, 5 mM 4-(2-hydroxyethyl)-1-piperazineethane sulphonic acid, pH 7.4.

0006-291X/85 $1.50

state of mitochondrial pyridine nucleotides (5,6) and hydroperoxides (7,8) have been implicated in the Ca^{2+} release mechanism from liver mitochondria it appeared of interest to investigate if divicine would stimulate Ca^{2+} release from mitochondria. The present report shows this to be the case.

MATERIAL AND METHODS

Vicine was obtained from Fluka, Buchs, Switzerland. Divicine was prepared daily from vicine by hydrolysis in 1 N HCl as described by Benatti et al. (4). The hydrolysate was subsequently neutralized with 1 N KOH. Isolation of mitochondria, Ca^{2+} transport studies, intramitochondrial hydrolysis of pyridine nucleotides, and determination of $\Delta\Psi$ were performed as described (8). Selenium-deficient mitochondria were isolated from selenium-deficient animals (7). Glutathione reductase activity was determined in mitochondrial extracts according to Ekloew et al. (9) in the absence of Triton X-100 and with 0.1 mg of protein/ml. To obtain extracts, mitochondria were incubated for 30 min in MSH buffer containing 5 uM rotenone, 2.5 mM K^+-succinate with or without BCNU. Subsequently the mitochondrial suspension was diluted 5-fold with cold MSH buffer and washed by centrifugation to remove unreacted BCNU. Mitochondria were then disrupted by sonication and centrifuged for 10 min at 10 000 x g. The supernatant was used for determination of the enzyme's activity.

RESULTS

Fig. 1 shows divicine-induced release of Ca^{2+} from energized rat liver mitochondria. The time of onset and the rate of Ca^{2+} release depend on the amount of divicine added (Fig. 1A) and the amount of Ca^{2+} previously accumulated (Fig. 1B) by mitochondria. In the presence of ruthenium red, a specific inhibitor of the mitochondrial Ca^{2+} uptake pathway (10), net release of Ca^{2+} is observed (Fig. 1C) immediately after addition of divicine. In selenium-deficient mitochondria Ca^{2+} release is greatly retarded as compared with control mitochondria (Fig. 1D).

Of the host of compounds known to induce Ca^{2+} release from mitochondria only few do so without damaging mitochondria. Measurements of the membrane potential, $\Delta\Psi$, give conclusive evidence for the intactness of mitochondria (11,12). Fig. 2 shows that upon addition of Ca^{2+} to energized mitochondria there is a sudden drop

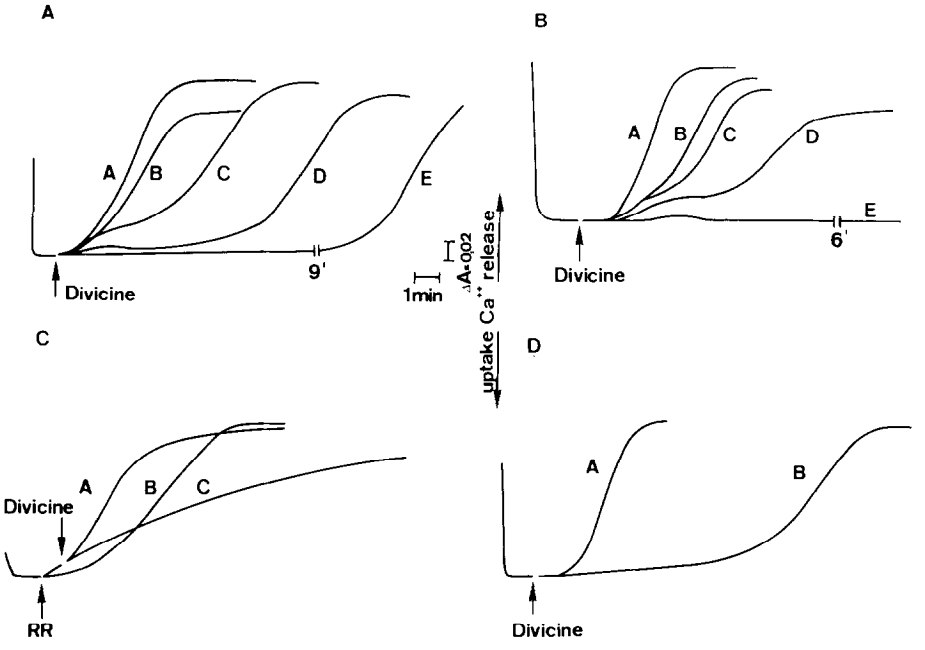

Fig. 1. Divicine-induced release of Ca^{2+} from mitochondria . A: Dependence on dose of divicine (A, 1mM; B, 0.75 mM; C, 0.5 mM; D, 0.25 mM; E, 0.1 mM); Ca^{2+} load was 56 nmol/mg of protein. B: Dependence on Ca^{2+} load (A, 58 nmol; B, 46 nmol; C, 37 nmol; D, 29 nmol; E, 21 nmol/mg of protein, respectively); divicine was 1 mM. C: Effect of ruthenium red (RR) (A: 4 μM RR, and 1 mM divicine added at the second arrow; B: 1 mM divicine; C: 4 μM RR); Ca^{2+} load was 55 nmol/mg of protein. D: Ca^{2+} release in selenium-adequate (A) and selenium-deficient (B) mitochondria; divicine was 1 mM, Ca^{2+} load was 57 nmol/mg of protein.

in $\Delta\Psi$ due to the energy-requiring sequestration of Ca^{2+} by mitochondria. When Ca^{2+} uptake is complete mitochondria are again fully energized as shown by $\Delta\Psi$—values around 190 mV. Addition of divicine to these mitochondria results in a gradual decrease of

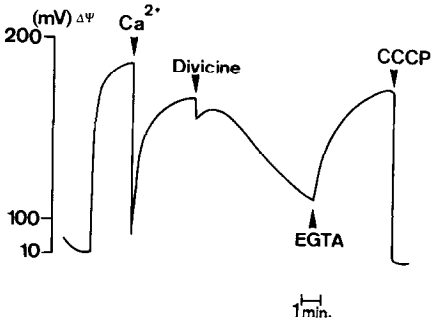

Fig. 2. Membrane potential of mitochondria measured with a tetraphenylphosphonium electrode. Ca^{2+} : 56 nmol/mg of protein; divicine: 1 mM; EGTA: 0.5 mM; CCCP: 1.5 μM.

$\Delta\Psi$ (Fig. 2). This decrease is, however, not due to damage of mitochondria since it can be prevented by the Ca^{2+}-chelator EGTA which prevents reuptake of Ca^{2+} and therefore a continuous and energy-draining cycling of Ca^{2+} (13) across the inner mitochondrial membrane. Addition of the uncoupler CCCP results in the expected complete collaps of $\Delta\Psi$ (Fig. 2).

Divicine has been reported to oxidize pyridine nucleotides (4). We did not observe non-enzymatic oxidation of pyridine nucleotides when mixing them with divicine. In mitochondria, however, we observed divicine-induced pyridine nucleotide oxidation when measured spectrophotometrically at 340-370 nm (results not shown). In Ca^{2+}-loaded mitochondria oxidized pyridine nucleotides can be enzymatically hydrolyzed at the β-N-glycosidic bond linking the (ADP)-ribose and nicotinamide part (8). A possible intramitochondrial hydrolysis of pyridine nucleotides induced by divicine was assayed by measurements of the release of pyridine nucleotide-derived nicotinamide from mitochondria (8). Fig. 3 shows that divicine indeed causes intramitochondrial pyridine nucleotide hydrolysis the rate and extent of which are dependent on the amount of Ca^{2+} previously accumulated by mitochondria. At a load of 55 nmol/mg of mitochondrial protein 0.5 and 1.0 mM divicine is equally effective. At 18 nmol Ca^{2+}/mg of protein hydrolysis induced by 1 mM divicine is much less pronounced.

Autooxidation of divicine results in the formation of H_2O_2 (4). Oxygen consumption by divicine concomitant with H_2O_2 formation is shown with an oxygen electrode in combination with catalase (Fig. 4, curve A and B). A possible redox cycling of divicine by mitochondria was similarly investigated. In the presence of cyanide, i.e. when respiration of mitochondria is completely blocked, divicine causes a transient and massive oxygen consumption

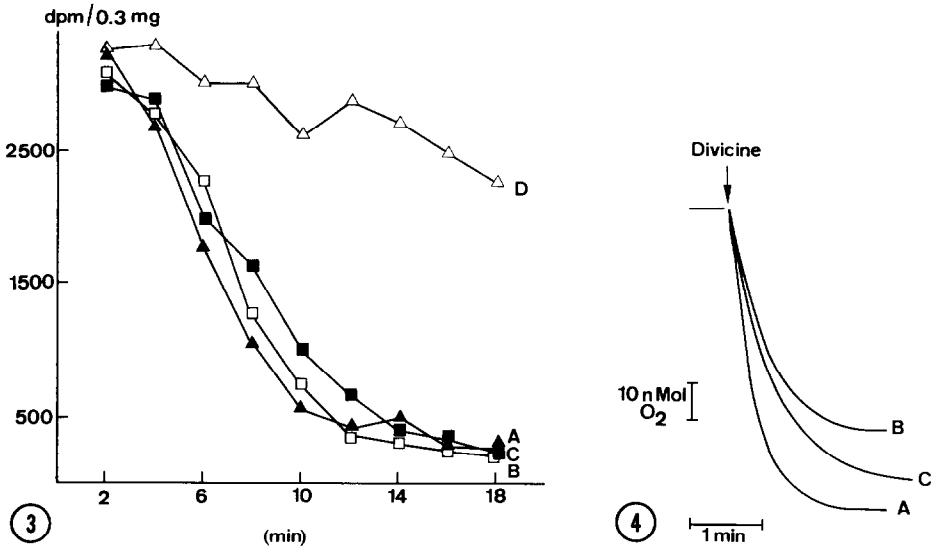

Fig. 3. Intramitochondrial hydrolysis of pyridine nucleotides. Pyridine nucleotides were radioactively labeled in vivo at the nicotinamide moiety (3300 dpm/0.3 mg of protein), and release of nicotinamide from mitochondria upon hydrolysis was assayed by Millipore filtration. A: 1 mM divicine , Ca^{2+} load 55 nmol/mg of protein; B: 0.5 mM divicine, Ca^{2+} load 55 nmol/mg of protein; C: 1 mM divicine , Ca^{2+} load 37 nmol/mg of protein; D: 1 mM divicine, Ca^{2+} load 18 nmol/mg of protein.

Fig. 4. Oxygen consumption induced by divicine in the presence of 0.2 mM cyanide measured in a Clark-type electrode. A: 1 mM divicine ; B: 1 mM divicine plus 40 µg bovine liver catalase; C: 1 mM divicine plus mitochondria, 2 mg protein/ml, in the presence of 2.5 mM succinate and 5 µM rotenone. All measurements were performed in MSH buffer, pH 7.4.

(Fig. 4, curve C). The amount of oxygen consumed is significantly larger than that seen with divicine and catalase indicating redox cycling of divicine by mitochondria. It should be noted that our mitochondrial preparation contains significant amounts of peroxisomal catalase.

H_2O_2 induces Ca^{2+} release from mitochondria due to its metabolism via the enzyme cascade consisting of glutathione peroxidase (EC 1.11.1.9), glutathione reductase (EC 1.6.4.2), and the energy-linked $NAD(P)^+$ transhydrogenase (EC 1.6.1.1) (7). Since, as shown above, divicine causes formation of H_2O_2 in mitochondria, and since divicine can oxidize GSH to GSSG (4), the involvement of glutathione peroxidase and glutathione reductase in the divicine-

induced Ca^{2+} release mechanism was investigated. To this end, mitochondria with very low glutathione peroxidase activity were isolated from selenium-deficient animals (7), or partial inhibition (35% activity remaining) of glutathione reductase was achieved by treatment of mitochondria with BCNU. As already noted divicine is much less effective in inducing Ca^{2+} release from selenium deficient mitochondria than from selenium-adequate mitochondria (c.f. Fig 1D). Treatment of mitochondria with BCNU dissolved in ethanol also slows, albeit only slightly, the divicine-induced Ca^{2+} release as compared with control mitochondria that had been treated with the vehicle ethanol alone (result not shown).

DISCUSSION

Several chemically unrelated compounds induce Ca^{2+} release from intact rat liver mitochondria, namely oxaloacetate or acetoacetate (5,6), hydroperoxides (7,8,12,14-16), menadione (17), and alloxan (18). The common denominator of their mode of action during Ca^{2+} release is oxidation and hydrolysis of pyridine nucleotides. Acetoacetate and oxaloacetate oxidize pyridine nucleotides at the level of the citric acid cycle; hydroperoxides via the glutathione enzyme cascade and $NAD(P)^+$ transhydrogenase ; menadione via the action of D,T-diaphorase (EC 1.6.99.2) (B. Frei and C. Richter, manuscript in preparation); and alloxan by redox cycling and direct oxidation (18). Oxidized pyridine nucleotides are then hydrolyzed by an intramitochondrial NAD^+ glycohydrolase (EC 3.2.2.5) (19, 20). Similar to the above-mentioned compounds, also the divicine-induced Ca^{2+} release occurs from intact mitochondria and is linked to pyridine nucleotides: it is accompanied by their oxidation and hydrolysis. The mode of action of divicine is probably several-fold. Firstly, as shown by measurements of

oxygen consumption, divicine leads to the formation of H_2O_2 by autooxidation and redox cycling in mitochondria. H_2O_2 then oxidizes pyridine nucleotides as discussed above. This is borne out by the sensitivity of the divicine-induced Ca^{2+} release to selenium-deficiency and to inhibition of glutathione reductase. Secondly, since divicine can oxidize GSH to GSSG, glutathione reductase-mediated pyridine nucleotide oxidation may also be initiated by such a divicine-induced shift of the glutathione redox state. The relative importance of H_2O_2 and the direct oxidation of glutathione is not clear at the moment. Thirdly, divicine may possibly induce intramitochondrial pyridine nucleotide oxidation non-enzymatically (4).

Disturbance of the cellular Ca^{2+} homeostasis leads to cell damage (21) and eventually to cell death (22). In hepatocytes a disturbed Ca^{2+} homeostasis is induced by t-butylhydroperoxide and menadione (22, 23) which deplete mitochondrial and microsomal Ca^{2+} stores and, as a consequence, increase the cytosolic free Ca^{2+} content. Since divicine induces Ca^{2+} release by basically the same mechanism and at concentrations that may be reached after ingestion of fava beans, damage by divicine of cells other than red blood cells can be expected.

ACKNOWLEDGEMENT

This work was supported by the Schweizerischer Nationalfonds, grant 3.503-0.83.

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