Gliotoxin Stimulates Ca^{2+} Release from Intact Rat Liver Mitochondria†

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ABSTRACT: Gliotoxin is an epidithiodioxopiperazine compound which can both react with sulfhydryl groups and form hydrogen peroxide. Rat liver mitochondria contain a prooxidant-regulated specific Ca^{2+} release pathway. Here we report that gliotoxin at low concentrations stimulates Ca^{2+} release via this pathway in isolated mitochondria. Ca^{2+} release is not promoted by gliotoxin exposed to disulfide-reducing reagents prior to addition to mitochondria or when its disulfide moiety is dimethylated. Gliotoxin is equally effective in glutathione-depleted and glutathione-adequate mitochondria. This and the unchanged mitochondrial oxygen consumption in the presence of gliotoxin suggest that the compound stimulates Ca^{2+} release by reacting with critical mitochondrial thiol compounds and not by increasing hydrogen peroxide formation in mitochondria. The gliotoxin-induced Ca^{2+} release is paralleled by hydrolysis of mitochondrial pyridine nucleotides, and both pyridine nucleotide hydrolysis and Ca^{2+} release are inhibited by cyclosporin A. These findings provide further insight into the regulation of Ca^{2+} release from intact mitochondria.

The control of Ca^{2+} uptake and release is important for proper mitochondrial functioning since several dehydrogenases and nucleic acid metabolism in mitochondria are regulated by Ca^{2+} [for recent reviews, see Gunter and Pfeiffer (1990) and Richter (1992)]. Mitochondrial Ca^{2+} transport also contributes to cellular Ca^{2+} homeostasis. Given their large Ca^{2+} storage capacity, the organelles provide a safety device against Ca^{2+} flooding of the cytosol, and they sense locally generated domains of high cytosolic Ca^{2+} concentrations (Rizzuto et al., 1993). Mitochondrial uptake and release of Ca^{2+} occur via different pathways (Carafoli, 1979). The former is driven by the electrochemical potential ($\Delta\psi$)[1] built up across the inner mitochondrial membrane. The latter is observed under two conditions: Release occurs either with preservation of $\Delta\psi$ and is specific for Ca^{2+} or when $\Delta\psi$ collapses and the inner mitochondrial membrane becomes nonspecifically leaky. In rat liver mitochondria, the specific release pathway depends on the hydrolysis of oxidized pyridine nucleotides and is stimulated by prooxidants (Richter, 1992). Despite much effort, no components engaged in mitochondrial Ca^{2+} transport are identified with certainty at the molecular level.

Gliotoxin (GT) (Figure 1) is a hydrophobic fungal metabolite of the epipolythiodioxopiperazine group whose characteristic is a quinoid moiety and a disulfide bridge across the piperazine ring (Waring et al., 1988). GT is an antiphagocytic and immunomodulating agent (Müllbacher et al., 1985) that can block membrane thiols (Jones & Hancock, 1988). It also causes DNA fragmentation *in vitro* when incubated with reductants such as glutathione or NADH, and evidence has been presented that the GT-induced DNA damage is ultimately due to reactive oxygen species produced by cyclic reduction and oxidation (redox cycling) of GT (Eichner et al., 1988). We now report that GT stimulates Ca^{2+} release from intact isolated rat liver mitochondria. Our data suggest that the stimulation is most likely due to reaction of GT with thiols critically involved in the specific Ca^{2+} release pathway rather than to redox cycling of GT.

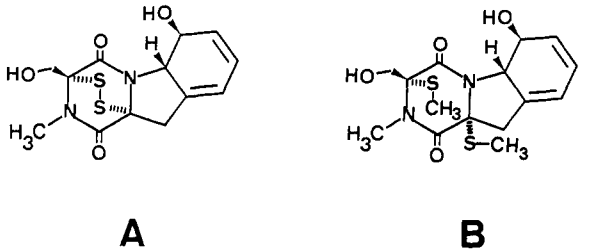

FIGURE 1: Structure of gliotoxin (A) and dimethyl-GT (B).

MATERIALS AND METHODS

Materials. Cyclosporin A (CSA) was a gift of Sandoz Pharma Preclinical Research, Basel, Switzerland. It was stored in solid form at −20 °C and dissolved in ethanol immediately prior to use. GT and didethiobis(methylthio)-gliotoxin (dimethyl-GT) were from Sigma, Buchs, Switzerland. They were dissolved in dimethyl sulfoxide prior to use. $^{45}Ca^{2+}$ and [*carboxyl*-^{14}C]nicotinic acid were from Amersham International, Buckinghamshire, U.K., and [^{14}C-(U)]sucrose was from New England Nuclear, Boston, MA. All other chemicals were purchased from standard suppliers and were of the highest purity available.

Isolation of Mitochondria. The isolation of rat liver mitochondria was performed by differential centrifugation as described (Schlegel et al., 1992). The protein content was determined by the biuret method with bovine serum albumin as standard.

Labeling of Mitochondrial Pyridine Nucleotides in Vivo. Overnight-fasted rats were injected intravenously with [*carboxyl*-^{14}C]nicotinic acid (12.5 μCi, 0.223 μmol) (Lötscher et al., 1980) in phosphate-buffered saline. After 3 h, the animals were killed, and liver mitochondria were isolated.

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[1] Abbreviations: CSA, cyclosporin A; $\Delta\psi$, electrical potential across the inner mitochondrial membrane, negative inside; dimethyl-GT, didethiobis(methylthio)gliotoxin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; GT, gliotoxin.

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Depletion of mitochondrial glutathione in vivo was performed as described (Traber et al., 1992). Briefly, after starvation overnight, the rats were injected i.p. with 250 mg of phorone (dissolved in sunflower oil)/kg of body weight. Mitochondria were isolated 3 h after phorone injection.

Standard Incubation Procedure. Mitochondria (2 mg of protein/mL) were incubated at 25 °C with continuous stirring and oxygenation in 3 mL of 210 mM mannitol, 70 mM sucrose, and 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.2.

Determination of Ca^{2+} Uptake and Release by Mitochondria. The standard incubation procedure was followed. After the addition of rotenone (5 μM) and K^+ succinate (2.5 mM), mitochondria were loaded with Ca^{2+}. Its movement across the inner mitochondrial membrane was monitored by the spectrophotometric or isotope technique (Millipore filtration) as described (Lötscher et al., 1980; Schlegel et al., 1992). The former was performed in the presence of 50 μM arsenazo III, the latter with $^{45}Ca^{2+}$. Ca^{2+} was added to give a total load as indicated in the figure legends, and its uptake was allowed to proceed for 2−3 min. At time 0 min as indicated in the figures, ruthenium red (2 nmol/mg of protein) and, 30 s later, GT were added.

Determination of Mitochondrial Membrane Potential. Mitochondria were incubated according to the standard procedure in the presence of 10 μM safranine. After the addition of rotenone (5 μM) and K^+ succinate (2.5 mM), they were loaded with Ca^{2+}. $\Delta\psi$ was determined in an Aminco DW2A spectrophotometer at 511−533 nm as described by Åkerman and Wikström (1976). Other compounds were added as indicated in Figure 3.

Spectrophotometric Analysis of Mitochondrial Pyridine Nucleotides. The standard incubation procedure was followed. After the addition of rotenone (5 μM) and K^+ succinate (2.5 mM), the absorption of mitochondrial pyridine nucleotides was determined in an Aminco DW2A spectrophotometer at 340−370 nm (Schweizer et al., 1993). Other compounds were added as indicated in Figure 5.

Determination of Intramitochondrial Pyridine Nucleotide Hydrolysis. Rats were injected intravenously with [*carboxyl*-^{14}C]nicotinic acid (see above). Mitochondria isolated from their livers were incubated according to the standard procedure in the presence of rotenone (5 μM) and K^+ succinate (2.5 mM) (except for Figure 6, trace c). At time 0 min, ruthenium red (2 nmol/mg of protein) and, 30 s later, GT were added. Release of nicotinamide as an indicator of pyridine nucleotide hydrolysis (Lötscher et al., 1980) was followed with the Millipore filtration technique (Schlegel et al., 1992). In order to deplete mitochondria of Ca^{2+} (Figure 6, trace c), they were incubated for 2 min in the presence of 5 mM rotenone. After the addition of 5 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), mitochondria were energized with 2.5 mM K^+ succinate. Other compounds were added as indicated in Figure 6.

Sucrose Entry. Sucrose entry into mitochondria was followed by the isotope technique in combination with filtration. Mitochondria were incubated according to the standard procedure in the presence of [^{14}C(U)]sucrose (0.3 mCi/mL) and energized with K^+ succinate in the presence of rotenone. Aliquots of 150 mL were withdrawn at the times indicated in the legend to Figure 4 and analyzed for radioactivity with the Millipore filtration technique described above.

Incubation of Gliotoxin with Sulfhydryl Reagents. GT was incubated with a 10-fold excess of reduced glutathione, dithiothreitol, or *N*-acetylcysteine for 20 min at 37 °C prior to addition to mitochondria.

Oxygen uptake was measured with a Clark-type electrode as described (Frei et al., 1985).

RESULTS

Stimulation by Gliotoxin of Ca^{2+} Release. GT stimulates Ca^{2+} release from mitochondria as shown by the spectrophotometric (Figure 2A,B) and the isotope technique (Figure 2C). The stimulation is transient, and its magnitude depends on the quantity of GT added to mitochondria. Concentrations as low as 2 μM elicit the release. A total of 1 μM cyclosporin A (CSA) completely inhibits the release induced by 30 μM GT.

Intactness of Mitochondria during Gliotoxin-Induced Ca^{2+} Release. The determination of $\Delta\psi$ is useful to assess the intactness of mitochondria during and after Ca^{2+} release (Baumhüter & Richter, 1982). When GT is added to mitochondria loaded with Ca^{2+} (45 nmol/mg of protein), $\Delta\psi$ changes depend on whether or not mitochondria are allowed to continuously release and take up (cycle) Ca^{2+} (Figure 3). Thus, $\Delta\psi$ decreases soon after the addition of GT in the absence of the Ca^{2+} uptake inhibitor ruthenium red (trace b), but increases in its presence (trace a), *i.e.*, under conditions which prevent Ca^{2+} cycling. Next to $\Delta\psi$, sucrose entry into mitochondria is a useful parameter to analyze nonspecific permeability changes in the inner mitochondrial membrane (Al-Nasser & Crompton, 1986). Figure 4 shows that sucrose does not enter mitochondria upon the addition of GT to the Ca^{2+}-loaded organelles provided Ca^{2+} cycling is prevented. Also, no GT-induced mitochondrial swelling (Hadler et al., 1973) was observed under these conditions (results not shown).

Mode of Action of Gliotoxin. Ca^{2+} release from intact rat liver mitochondria is evoked by a variety of prooxidants including hydrogen peroxide (Lötscher et al., 1980) or by phenylarsine oxide (Schweizer et al., 1994), a lipophilic compound known to react with vicinal protein thiols. Since GT in the presence of reductants can form hydrogen peroxide (Eichner et al., 1988) but can also react with thiols (Jones & Hancock, 1988), it was of interest to gain insight into the mechanism by which GT stimulates mitochondrial Ca^{2+} release. The following observations (results not shown) rule out significant redox cycling of GT in mitochondria: (i) There was no extra oxygen consumption induced by GT when re-uptake of the released Ca^{2+} was prevented by EGTA or ruthenium red. (ii) Oxygen consumption by mitochondria in the presence of succinate and 100 μM KCN (Frei et al., 1985) was not increased with up to 200 μM GT. (iii) Glutathione-depleted mitochondria (glutathione content <0.1 nmol/mg of protein) (Traber et al., 1992) showed a very similar response as glutathione-adequate mitochondria with respect to the kinetics and extent of the GT-induced Ca^{2+} release, whereas 500 μM *tert*-butyl hydroperoxide was unable to mobilize Ca^{2+} from glutathione-depleted mitochondria.

On the other hand, after a 20-min preincubation with the disulfide-reducing reagents glutathione, dithiothreitol, or *N*-acetylcysteine, 30 μM GT did not elicit Ca^{2+} release, whereas simultaneous addition of GT and reducing agents did not abolish the GT-induced Ca^{2+} mobilization. Further-

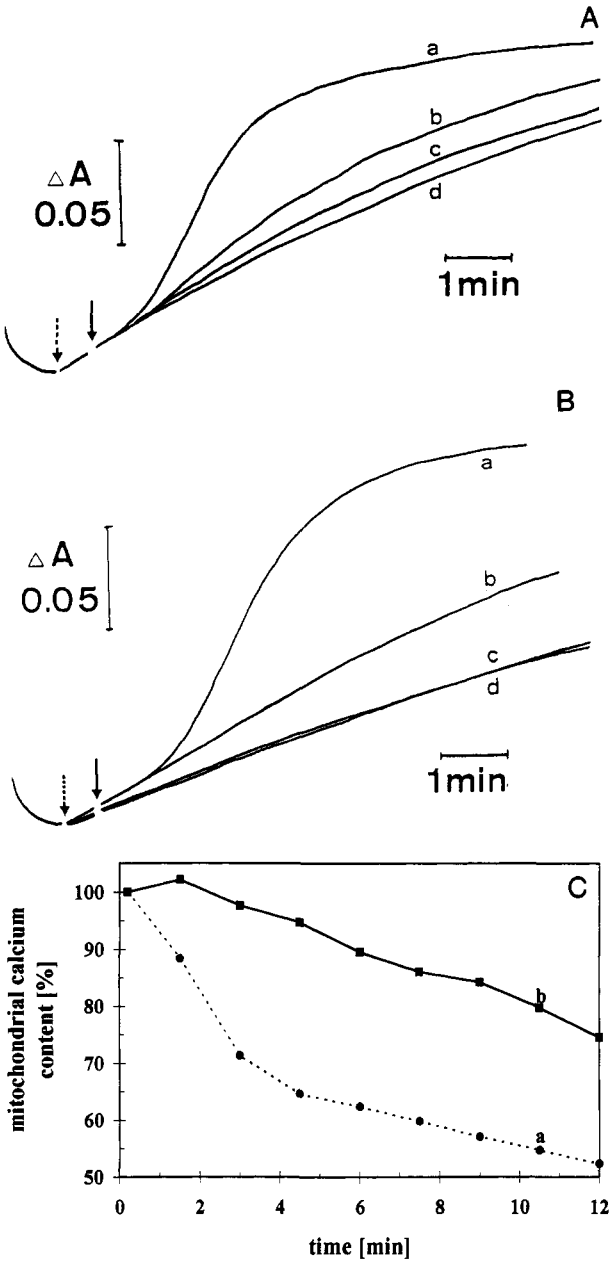

FIGURE 2: Gliotoxin-stimulated Ca^{2+} release from rat liver mitochondria. Mitochondria were incubated according to the standard procedure. After the addition of rotenone and K^+ succinate, they were loaded with 45 nmol of Ca^{2+}/mg of protein. (A) Spectrophotometric assay. At the dashed arrow, ruthenium red (2 nmol/mg of protein), and at the solid arrow, 15 μM (trace a), 5 μM (trace b), or 2 μM GT were added; trace d, vehicle alone. (B) Spectrophotometric assay. Mitochondria were incubated in the presence (traces c and d) or the absence (traces a and b) of 1 μM CSA. At the dashed arrow, ruthenium red (2 nmol/mg of protein), and at the solid arrow, 30 μM GT (traces a and c) or its vehicle (traces b and d) were added. (C) Isotope technique. After the addition of rotenone and K^+ succinate, mitochondria were loaded with Ca^{2+}, and 2 min thereafter (time 0 min) ruthenium red (2 nmol/mg of protein) and, 30 s later, 30 μM GT (trace a) or its vehicle (trace b) were added. The results shown are from one experiment typical of five.

more, 30 μM dimethyl-GT, which does not have a disulfide group (Figure 1), did not cause Ca^{2+} release (results not shown).

Since Ca^{2+} release from intact mitochondria is casually linked to the hydrolysis of intramitochondrial oxidized pyridine nucleotides (Richter & Kass, 1991), we determined

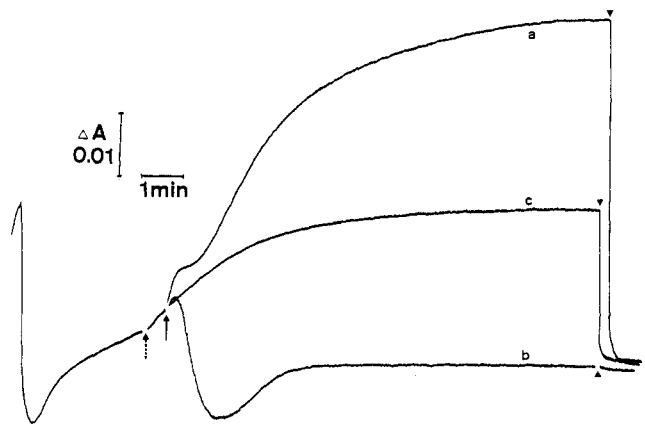

FIGURE 3: Gliotoxin-induced alterations of the mitochondrial membrane potential. Mitochondria were incubated according to the standard procedure in the presence of 10 μM safranine and rotenone and were energized with K^+ succinate. 3 min after the addition of Ca^{2+} (45 nmol/mg of protein), ruthenium red (2 nmol/mg of protein) (dashed arrow) (traces a and c) and, 30 s later, 30 μM GT (traces a and b) or its vehicle (trace c), were added (solid arrow). At the triangles, the uncoupler carbonylcyanide-3-chlorophenylhydrazone (1 μM) was added. The results shown are from one experiment typical of five.

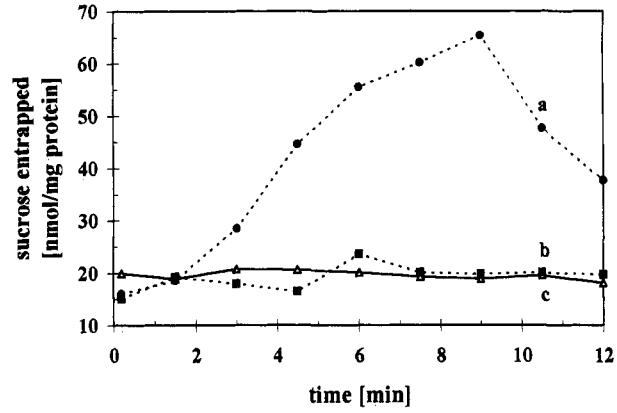

FIGURE 4: Gliotoxin-induced sucrose entry into mitochondria in the presence or absence or ruthenium red. Mitochondria were incubated according to the standard procedure and energized with K^+ succinate in the presence of rotenone. 2 min after the addition of Ca^{2+} (45 nmol/mg of protein), ruthenium red (2 nmol/mg of protein) (time 0 min, traces b and c) and, 30 s later, 30 μM GT (traces a and b) or its vehicle (trace c) were added. Samples were analyzed for sucrose entry as described in the Methods section. The results shown are from one experiment typical of three.

spectrophotometrically (Figure 5) their GT-dependent oxidation and hydrolysis. GT evokes a transient decrease of pyridine nucleotide absorption measured at 340−370 nm in Ca^{2+}-loaded, glutathione-adequate mitochondria (Figure 5A). The decrease is completely reversible in the presence (trace a) but incompletely reversible in the absence (trace b) of 1 μM CSA. In glutathione-deficient mitochondria (Figure 5B), GT induces little decrease of pyridine nucleotide absorption in the absence (trace b) and even less decrease in the presence of 1 μM CSA (trace a). The direct determination of pyridine nucleotide hydrolysis, assayed by nicotinamide release from mitochondria, reveals that GT stimulates it in a Ca^{2+}-dependent manner (Figure 6). Thus, hydrolysis is not observed in Ca^{2+}-depleted mitochondria (trace c) but occurs in Ca^{2+}-loaded mitochondria (trace a) with the same time course as the GT-induced Ca^{2+} release (cf. Figure 2C). Hydrolysis is completely inhibited by 1 μM CSA (Figure 6,

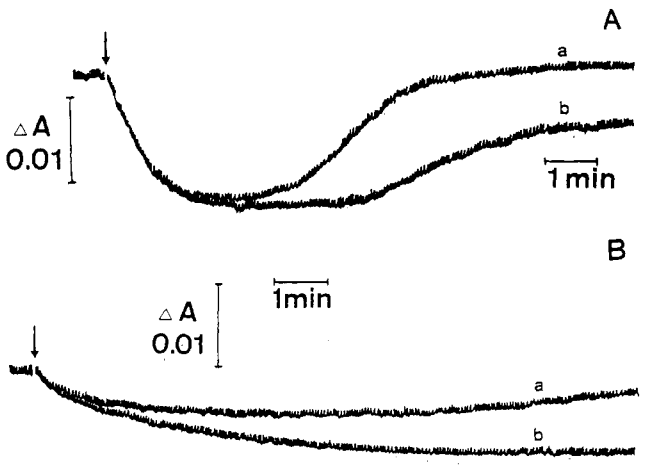

FIGURE 5: Gliotoxin-induced oxidation and re-reduction of mitochondrial pyridine nucleotides. Mitochondria were incubated according to the standard procedure in the presence (traces a) or absence (traces b) of 1 μM CSA. Changes in the redox state of mitochondrial pyridine nucleotides were monitored at 340−370 nm. After the addition of rotenone and K^+ succinate, mitochondria were loaded with 40 nmol of Ca^{2+}/mg of protein. 2 min after Ca^{2+} uptake, ruthenium red (2 nmol/mg of protein) and, 30 s later, 30 μM GT (arrow) were added. (A) Glutathione-adequate mitochondria, obtained from control animals. (B) Glutathione-deficient mitochondria, obtained from phorone-treated animals as described in the Methods section. The results shown are from one experiment typical of three.

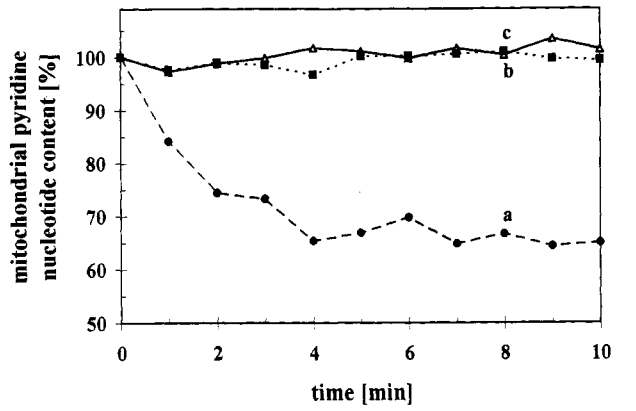

FIGURE 6: Gliotoxin-induced hydrolysis of mitochondrial pyridine nucleotides. Mitochondria labeled at the nicotinamide moiety were incubated according to the standard procedure. They were energized with rotenone and K^+ succinate (except for trace c) in the presence (trace b) or absence (traces a and c) of 1 μM CSA and loaded with 45 nmol of Ca^{2+}/mg of protein. At time 0 min, ruthenium red (2 nmol/mg of protein) and, 30 s later, 30 μM GT were added. Trace c: To deplete the mitochondrial Ca^{2+}, 2 min after the addition of rotenone, 5 mM EGTA and, 30 s thereafter, K^+ succinate were added. After another 2 min, 30 μM GT was added (time 0.5 min). Hydrolysis of pyridine nucleotides was determined by analysis of nicotinamide release with Millipore filtration. The vehicles alone did not cause pyridine nucleotide hydrolysis. The results shown are from one experiment typical of three.

trace b). In glutathione-depleted mitochondria, pyridine nucleotide hydrolysis as measured by nicotinamide release was somewhat slower but as extensive as in glutathione-adequate mitochondria (not shown).

DISCUSSION

GT has the potential both to form hydrogen peroxide and to act as a thiol-modifying reagent. Indeed, in the presence of reductants it produces DNA-damaging reactive oxygen species, among them hydrogen peroxide (Jones & Hancock, 1988), and it blocks membrane thiol groups (Eichner et al., 1988). Prooxidants such as hydroperoxides (Lötscher et al., 1979), menadione (Frei et al., 1986), alloxan (Frei et al., 1985), divicine (Graf et al., 1985), *N*-acetyl-*p*-benzoquinone imine (Weis et al., 1992), or 6-hydroxydopamine (Reichman et al., 1994) are well-established inducers of Ca^{2+} release from intact rat liver mitochondria. These compounds lead to an oxidation of intramitochondrial NADH, which is subsequently hydrolyzed in a Ca^{2+}-dependent manner to ADP ribose and nicotinamide. Circumstantial evidence strongly suggets protein (mono)ADP ribosylation in mitochondria to be the ultimate trigger for Ca^{2+} release [reviewed in Richter (1992)]. There are also indications that protein thiols are critically involved in the regulation of cation fluxes through the inner mitochondrial membrane (Siliprandi et al., 1978; Valle et al., 1993). For example, phenylarsine oxide modifies vicinal thiol groups in proteins and promotes the movement of ions, among them Ca^{2+}, through the inner mitochondrial membrane (Novgorodov et al., 1987; Lenartowicz et al., 1991; Bernardi, 1992) and stimulates the NAD^+-linked Ca^{2+} release from rat liver mitochondria (Schweizer et al., 1994).

We find that GT transiently stimulates Ca^{2+} release from rat liver mitochondria. The organelles remain intact during and after the release, provided Ca^{2+} cycling is prevented. This is concluded from the observations that during the GT-induced Ca^{2+} release, mitochondria in the presence of ruthenium red do not lose but rather rebuild $\Delta\psi$, do not allow sucrose to enter into their matrix, and do not swell. These findings unequivocally show an activation by GT of a Ca^{2+}-specific release pathway. This pathway is effectively blocked by CSA, which prevents the pyridine nucleotide-linked Ca^{2+} release from intact mitochondria (Schweizer et al., 1993).

GT is not measurably redox-cycled in mitochondria under conditions where it induces Ca^{2+} release. Even at a level 7-fold above the highest concentration used to induce release, no additional oxygen consumption by mitochondria was observed, indicating that the compound does not stimulate hydrogen peroxide production in mitochondria. Also, GT is equally effective as a Ca^{2+} mobilizer in glutathione-adequate as in glutathione-deficient mitochondria, in contrast to the hydroperoxide-induced Ca^{2+} release, which is very responsive to the mitochondrial glutathione status (Traber et al., 1992; Bellomo et al., 1984). This rules out a significant involvement of the enzyme cascade glutathione peroxidase/glutathione reductase/energy-linked transhydrogenase (Lötscher et al., 1979) in the GT-dependent Ca^{2+} release. In addition, GT but not dimethyl-GT causes Ca^{2+} release, and GT treated with thiol-reducing reagents loses its effectiveness. It thus appears that it is the disulfide bridge of GT which is required for mobilization of Ca^{2+} from mitochondria.

A decrease of pyridine nucleotide absorption measured at 340−370 nm reflects either their oxidation or hydrolysis of oxidized pyridine nucleotides followed by oxidation of some of the remaining reduced molecules due to thermodynamic equilibration. In glutathione-adequate mitochondria, GT causes a pronounced decrease in absorption followed by an incomplete re-reduction in the absence but a complete re-reduction in the presence of CSA, which prevents pyridine nucleotide hydrolysis (Richter et al., 1990). In glutathione-deficient mitochondria, there is only a little absorption

decrease in the absence of CSA and an even smaller decrease in the presence of CSA. The extent of pyridine nucleotide hydrolysis, judged from the absorption difference in the presence and absence of CSA at the end of the GT-induced Ca^{2+} release, does not depend on the mitochondrial glutathione status. The pronounced absorption decrease reflects glutathione oxidation by GT, followed by enzymatic reduction of oxidized glutathione at the expense of NAD(P)H (unpublished observation) and hydrolysis of $NAD(P)^+$. The GT-induced disappearance of pyridine nucleotide absorption in glutathione-deficient mitochondria suggests a GT-stimulated hydrolysis of oxidized pyridine nucleotides followed by pyridine nucleotide redox equilibration. It should be noted that between 14 and 43% of pyridine nucleotides are oxidized in Ca^{2+}-loaded mitochondria in the presence of rotenone and succinate (Lötscher et al., 1979; Frei and Richter, unpublished result).

The measurement of nicotinamide release confirms the spectrophotometric pyridine nucleotide analysis. Thus, nicotinamide release is inhibited by CSA and is somewhat faster in glutathione-adequate mitochondria, where GT causes the formation of oxidized glutathione followed by NAD(P)H oxidation.

Our results show that the specific Ca^{2+} release from rat liver mitochondria is possible when thiols other than glutathione are modified in such a way that hydrolysis of oxidized pyridine nucleotides is achieved. Such thiols may reside on a protein catalyzing pyridine nucleotide hydrolysis (*e.g.*, a NAD^+ glycohydrolase) or on a molecule regulating such an enzyme. Hydrogen peroxide, produced by the mitochondrial respiratory chain under physiological and pathophysiological conditions (Chance et al., 1979; McCord, 1993), oxidizes mitochondrial pyridine nucleotides (Lötscher et al., 1979, 1980). It is also likely to influence at least indirectly the redox state of thiols. Hydrogen peroxide may, therefore, act as a natural regulator of the mitochondrial and cellular Ca^{2+} homeostasis.

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