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FURTHER CHARACTERIZATION OF THE EVENTS INVOLVED IN MITOCHONDRIAL Ca^{2+} RELEASE AND PORE FORMATION BY PROOXIDANTS

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Abstract—Addition of the prooxidant 3,5-dimethyl-*N*-acetyl-*p*-benzoquinone imine ($3,5(Me)_2$NAPQI) to Ca^{2+}-loaded mitochondria caused a rapid and extensive release of the sequestered Ca^{2+}. Ca^{2+} release was accompanied by irreversible NAD(P)H oxidation and was followed by the release of adenine and pyridine nucleotides into the extramitochondrial medium; this is evidence of the opening of the pore in the inner mitochondrial membrane. Preincubation of the mitochondria with ADP, cyclosporin A (CSA), *m*-iodobenzylguanidine (MIBG) or Mg^{2+} inhibited the prooxidant-induced Ca^{2+} release and prevented pore-opening. When mitochondria were preincubated with ruthenium red, Ca^{2+} release was only minimally stimulated by $3,5(Me)_2$NAPQI. However, increasing the concentration of the prooxidant caused release of an increasing fraction of the sequestered Ca^{2+}. Alternatively, increasing the intramitochondrial Ca^{2+} load resulted in a lowering of the concentration of $3,5(Me)_2$NAPQI required for near complete Ca^{2+} release to occur. In the presence of ruthenium red, $3,5(Me)_2$NAPQI-induced Ca^{2+} release was accompanied by irreversible pyridine nucleotide oxidation and followed by the release of nucleotides into the extramitochondrial medium, events which were prevented on preincubation with CSA. Similarly, the addition of CSA, ADP or MIBG during $3,5(Me)_2$NAPQI-induced Ca^{2+} release arrested further Ca^{2+} release. In addition to their inhibitory effect on the $3,5(Me)_2$NAPQI-induced Ca^{2+} release, CSA, ADP or MIBG also decreased the rate of the basal, ruthenium red-induced mitochondrial Ca^{2+} release by 45–70%. It is proposed that the basal, ruthenium red-induced and the prooxidant-induced mitochondrial Ca^{2+} release occur through a common component that is sensitive to inhibition by CSA, ADP and MIBG and that is involved in mitochondrial pore formation. Furthermore, $3,5(Me)_2$NAPQI-induced pore opening does not involve Ca^{2+}-cycling, but rather involves a site(s) that is (are) synergistically activated by Ca^{2+} and the prooxidant.

Key words: mitochondria; calcium; oxidative stress; pore; cyclosporin A; pyridine nucleotides

Intramitochondrial Ca^{2+} homeostasis is controlled by separate Ca^{2+}-uptake and release pathways. Uptake of Ca^{2+} into the mitochondrial matrix occurs through a ruthenium red-sensitive uniporter and is driven by the electrochemical transmembrane potential generated during respiration [1]. Release of Ca^{2+} from mitochondria may involve several distinct pathways [2]. Thus, under physiological conditions efflux of Ca^{2+} occurs in exchange for Na^+ or with H^+, the relative contribution of H^+ versus Na^+ depending on the tissue of origin of mitochondria. However, evidence has been presented to suggest that under pathological conditions such as oxidative stress from exposure to prooxidants or during the reoxygenation of hypoxic tissue, excessive mitochondrial Ca^{2+} uptake and subsequent Ca^{2+} loss occur [3–5]. Ca^{2+} release may then be a non-specific event, possibly due to opening of a pore of about 20 Å diameter in the inner mitochondrial membrane [6–11]. Pore opening has been intensively investigated in isolated mitochondria, and been shown to require relatively high intramitochondrial Ca^{2+} together with an inducing agent, such as phosphate or the prooxidant tBuOOH† [6–11]. This pore is probably identical with the permeability transition pore observed in mitochondrial swelling experiments [12–18], and has also been detected in patch clamp experiments [19]. Pore opening is fully reversed on Ca^{2+} removal [8, 10] and is potently inhibited by the immunosuppressant cyclosporin A [9, 15, 16].

Recently, however, evidence has been presented to suggest that the prooxidant tBuOOH can also activate a selective pathway of Ca^{2+} efflux from mitochondria without pore opening if Ca^{2+} reuptake is prevented by ruthenium red or EGTA. Thus, in the case of tBuOOH, pore opening appears to be an event that is secondary to specific Ca^{2+} release and is caused by Ca^{2+}-cycling [20].

The aim of the present study was to further investigate the early and critical event(s) triggering prooxidant-induced Ca^{2+} release. We show here that Ca^{2+} release induced by the prooxidant

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† Abbreviations: tBuOOH, *tert*-butyl hydroperoxide; Arsenazo III, 2,2'-(1,8-dihydroxy-3,6-disulphonaphthalene-2,7-bisazo)-bis-(benzene arsonic acid); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Me_2SO, dimethylsulfoxide; TCA, trichloroacetic acid; MIBG, *meta*-iodobenzylguanidine; $3,5(Me)_2$NAPQI, 3,5-dimethyl-*N*-acetyl-*p*-benzoquinone imine; $3,5(Me)_2$NAPQIH$_2$, 3,5-dimethyl-*N*-acetyl-*p*-aminophenol; FCCP, carbonyl cyanide-*p*-trifluoromethoxy-phenylhydrazone; ruthenium red, $(Ru_3O_2(NH_3)_{14})Cl_6 \cdot 4H_2O$.

3,5(Me)$_2$NAPQI, either in the absence or in the presence of ruthenium red, was inhibited by CSA, ADP, Mg^{2+} or MIBG. 3,5(Me)$_2$NAPQI-induced Ca^{2+} release was accompanied by pore opening, and pore opening was prevented by the same agents. CSA, ADP or MIBG also inhibited the basal, ruthenium red-induced Ca^{2+} release. We suggest that Ca^{2+} release observed under these different experimental conditions involves a common component that represents a structure involved in the mitochondrial pore. We furthermore show that 3,5(Me)$_2$NAPQI-induced pore opening does not require Ca^{2+}-cycling, but is synergistically triggered by the intramitochondrial Ca^{2+} load and the oxidant-concentration.

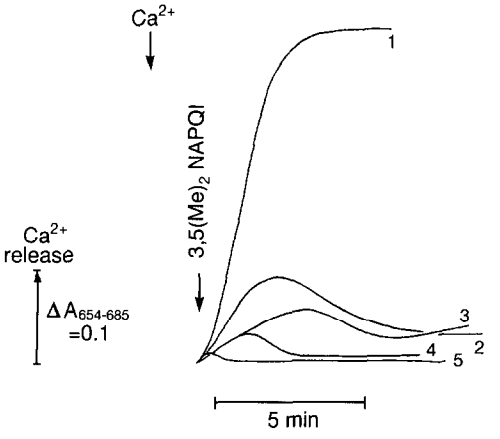

Fig. 1. Inhibitory effect of CSA, ADP, Mg^{2+} or MIBG on quinone imine-induced mitochondrial Ca^{2+} release. Mitochondria were loaded with Ca^{2+} and preincubated in the absence (trace 1) or presence of ADP (trace 2) (150 nmol/mg protein), MIBG (trace 3) (60 nmol/mg protein), Mg^{2+} (trace 4) (60 nmol/mg protein), or CSA (trace 5) (1.5 nmol/mg protein). The stimulation of Ca^{2+} release by 3,5(Me)$_2$NAPQI (90 nmol/mg protein, a concentration that resulted in near complete release of all sequestered Ca^{2+}), was measured as described in Materials and Methods. For clarity, trace 4 was corrected for the spectral interference produced by the Arsenazo III–Mg^{2+} complex. Traces are representative of three independent experiments conducted on separate mitochondrial preparations.

MATERIALS AND METHODS

Chemicals. 3,5(Me)$_2$NAPQI was obtained from Dalton Chemical Laboratories Inc. (Toronto, Ontario, Canada). MIBG and CSA were gifts from Dr L. Smets (The Netherlands Cancer Institute, Amsterdam, The Netherlands) and Sandoz (Basel, Switzerland), respectively. ADP, glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and glucose-6-phosphate were obtained from Boehringer (Mannheim, Germany). Arsenazo III (98% pure) and ruthenium red were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Cellulose-nitrate filters (0.45 μm pore size) were obtained from Sartorius (Göttingen, Germany). All other chemicals were of the highest purity commercially available.

Isolation of mitochondria. Liver mitochondria were isolated from male Wistar rats (ALAB AB, Sollentuna, Sweden; 200–250 g, fed *ad libitum*) as described previously [21]. The protein content of the final mitochondrial suspension was determined by the biuret method [22] using crystallized and lyophilized bovine serum albumin as standard.

Mitochondrial incubations. Unless stated otherwise, all incubations were performed in MSH buffer (mannitol (210 mM), sucrose (70 mM) and Hepes (3 mM), pH 7.1), at 25°. Mitochondria (1 mg protein/mL) were energized with succinate (5 mM) in the presence of rotenone (3 μM) for 2.5 min, before loading with Ca^{2+} (15 nmol/mg protein) for an additional 1.5 min. ADP, CSA, Mg^{2+} or MIBG was added 1 min after Ca^{2+}, or subsequent to 3,5(Me)$_2$NAPQI as described in the text. Ruthenium red was added 2 min after Ca^{2+}, and 3,5(Me)$_2$NAPQI was added (from concentrated stocks in dry Me_2SO prepared fresh every day) as described in the text. The final concentration of Me_2SO in the incubation mixtures never exceeded 0.3%.

Mitochondrial Ca^{2+} fluxes. Mitochondrial Ca^{2+} fluxes were measured by monitoring the change in absorbance of the metallochromophoric dye Arsenazo III (40 μM) using the wavelength pair 654–685 nm. The change in absorbance of 40 μM Arsenazo III linearly reflected the Ca^{2+} concentration up to a concentration of around 20 μM of Ca^{2+}.

Analysis of total and extramitochondrial pyridine nucleotides and adenine nucleotides. The mitochondrial pyridine nucleotide redox state was determined spectrophotometrically in a Shimadzu UV-3000 spectrophotometer using the wavelength pair 340–375 nm. In addition, the total and extramitochondrial adenine and oxidized pyridine nucleotide content was assayed by HPLC [23] as follows. Mitochondria (1 mg protein/mL) were energized and loaded with Ca^{2+} as described above. At selected time points, duplicate aliquots of 0.5 mL were then withdrawn from the mitochondrial incubations. One aliquot was directly added to 80 μL of 70% (v/v) PCA to determine total mitochondrial nucleotides. The other aliquot was filtered over a nitrocellulose filter into 80 μL of 70% (v/v) PCA to assay for released mitochondrial nucleotides. The samples were then processed for the analysis of pyridine nucleotides and adenine nucleotides as described in Ref. [23]. The nucleotides were separated using a CT-sil C_{18} column (5 μm, 250 × 4.6 mm) (Chrom Tech AB, Norsborg, Sweden) as reported previously [24].

In addition, extramitochondrial $NADP^+$ was measured spectrophotometrically using an enzymatic assay without prior separation of the mitochondria from the incubation medium. In this case, mitochondria (2 mg protein/mL) were loaded with Ca^{2+} (15 nmol/mg protein) and 3,5(Me)$_2$NAPQI-induced oxidation of $NAD(P)^+$ was allowed to proceed for 1.5 min before addition of CSA (1.5 nmol/mg) to cause pore closure. After $NAD(P)^+$ re-reduction in the presence of CSA was complete, glucose-6-phosphate (250 μM) and glucose-6-phosphate dehydrogenase (0.4 U/mL) were added to the mitochondrial incubation to reduce accessible (extramitochondrial) $NADP^+$.

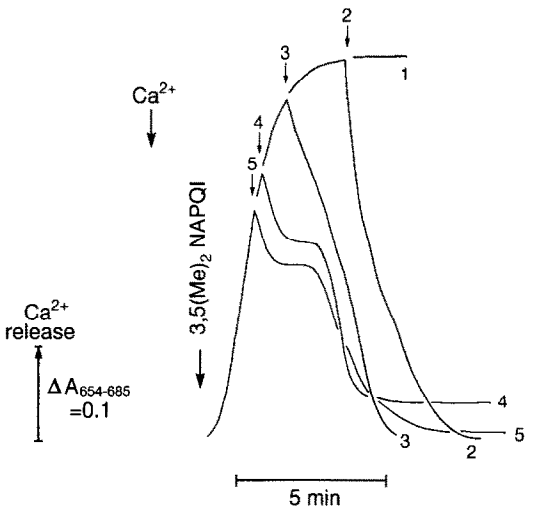

Fig. 2. CSA, ADP, Mg^{2+} or MIBG induces reuptake of Ca^{2+} by mitochondria after exposure to $3,5(Me)_2$NAPQI. Mitochondria were loaded with Ca^{2+} as described in Materials and Methods and release of all sequestered Ca^{2+} was induced by the addition of $3,5(Me)_2$NAPQI (90 nmol/mg protein) (trace 1). CSA (trace 2) (1.5 nmol/mg protein), ADP (trace 3) (150 nmol/mg protein), Mg^{2+} (trace 4) (60 nmol/mg protein) and MIBG (trace 5) (60 nmol/mg protein) were added where indicated by the arrows. Ca^{2+} release and reuptake was measured as described in Materials and Methods. Note that the change in absorbance of Arsenazo III linearly reflected the Ca^{2+} concentration up to around 20 μM. Traces are representative of three independent experiments conducted on separate mitochondrial preparations.

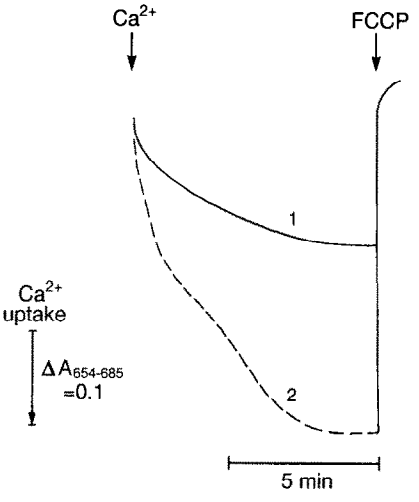

Fig. 3. Uptake of Ca^{2+} by control and $3,5(Me)_2$NAPQI-treated mitochondria. Mitochondria (1 mg protein/mL) were preincubated with CSA (1.5 nmol/mg) for 1 min and were subsequently diluted to a final protein concentration of 0.2 mg/mL by addition of a corresponding volume of MSH-buffer containing 15 μM Ca^{2+} and 40 μM Arsenazo III (final concentrations). Ca^{2+} uptake was measured at 685–654 nm, and a typical rate is shown in trace 1. Trace 2 shows the rate of CSA-induced reuptake of Ca^{2+} (after near complete release) by $3,5(Me)_2$NAPQI-treated mitochondria (1 mg protein/mL) using the same batch of mitochondria. FCCP (0.5 μM) was added as indicated by the right arrow. Traces are representative of three independent experiments conducted on separate mitochondrial preparations.

RESULTS

Mitochondrial Ca^{2+} release in the absence of ruthenium red and induction of Ca^{2+} reuptake by CSA, ADP, Mg^{2+} or MIBG

When Ca^{2+}-loaded mitochondria were incubated in the absence of ruthenium red, the addition of $3,5(Me)_2$NAPQI (90 nmol/mg protein) induced a rapid release of all the sequestered Ca^{2+}. Ca^{2+} release was inhibited by preincubating the mitochondria with either CSA, ADP, Mg^{2+} or MIBG (Fig. 1). In addition to their inhibitory effect on Ca^{2+} release when added before $3,5(Me)_2$NAPQI, these agents also induced complete reuptake of all the released Ca^{2+} by the mitochondria when added after exposure to $3,5(Me)_2$NAPQI (Fig. 2). CSA or ADP stimulated the reuptake of all the released Ca^{2+}, even after near complete discharge of the entire Ca^{2+} load from mitochondria (Fig. 2). By contrast, Mg^{2+} and MIBG induced total reuptake only when added before maximal Ca^{2+} release had occurred (Fig. 2). The observed reuptake suggests that the Ca^{2+} release pathway was operative in a reversible manner, and that ADP or CSA could induce its closure.

The criterion of reuptake as indicative of reversible opening of a Ca^{2+} release pathway has been questioned [2]. Since mitochondria can sequester large amounts of Ca^{2+}, the sequestration of all the released Ca^{2+} by a small subpopulation of mitochondria

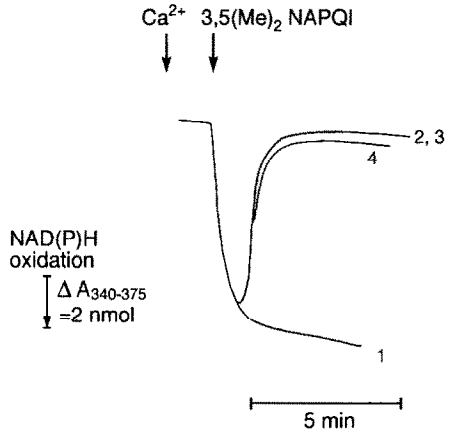

Fig. 4. Effect of CSA, ADP, or Mg^{2+} on $3,5(Me)_2$NAPQI-induced mitochondrial pyridine nucleotide oxidation. Mitochondria were loaded with Ca^{2+} and preincubated in the absence (trace 1) or presence of CSA (trace 2) (1.5 nmol/mg protein), Mg^{2+} (trace 3) (60 nmol/mg protein) or ADP (trace 4) (150 nmol/mg protein) before the addition of $3,5(Me)_2$NAPQI (90 nmol/mg protein). Pyridine nucleotide oxidation was measured as described in Materials and Methods. Traces are representative of three independent experiments conducted on separate mitochondrial preparations.

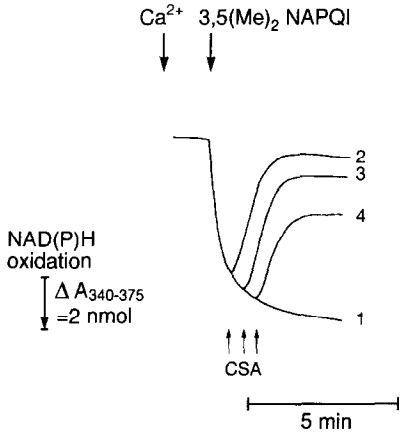

Fig. 5. Time-dependence of CSA-induced re-reduction of mitochondrial pyridine nucleotides when added after $3,5(Me)_2$NAPQI. Mitochondria were loaded with Ca^{2+} as described under Materials and Methods, and exposed to $3,5(Me)_2$NAPQI (90 nmol/mg protein) (trace 1). CSA (1.5 nmol/mg protein) was added 0.5 min (trace 2), 1 min (trace 3) or 1.5 min (trace 4) after $3,5(Me)_2$NAPQI as indicated by the arrows. Traces are representative of three independent experiments conducted on separate mitochondrial preparations.

whose Ca^{2+} release pathway has not been activated has been suggested to be indistinguishable from the reuptake by the whole population on closure of the release pathway [2]. Therefore we investigated whether a small subpopulation of control mitochondria could accumulate Ca^{2+} at a rate comparable to that observed with $3,5(Me)_2$NAPQI-treated mitochondria. Using a fraction that accounted for 20% of the total population, accumulation of Ca^{2+} in the presence of CSA was much slower than CSA-induced Ca^{2+} reuptake by $3,5(Me)_2$NAPQI-treated mitochondria (Fig. 3). Virtually identical results were obtained with ADP (data not shown). Around 40–50% of the total mitochondrial population were required to achieve a rate of Ca^{2+} uptake that was comparable to the rate of the CSA- or ADP-induced Ca^{2+} reuptake by $3,5(Me)_2$NAPQI-treated mitochondria (data not shown). It is unlikely that, after near complete release of Ca^{2+} from the whole mitochondrial population, in 40–50% of the mitochondria the Ca^{2+} release pathway would not have been activated. Consequently, the CSA- or ADP-induced reuptake of Ca^{2+} cannot be explained solely by the heterogeneity of the mitochondrial suspension. In contrast with CSA and ADP, Mg^{2+} or MIBG could induce reuptake of Ca^{2+} only when added before near complete Ca^{2+} release had occurred, and in this case both explanations, i.e. inactivation of the release pathway and reuptake of Ca^{2+} by a subpopulation need to be considered.

Mitochondrial NAD(P)H oxidation and release of mitochondrial nucleotides

In agreement with previous results [24, 25], $3,5(Me)_2$NAPQI-induced mitochondrial Ca^{2+} release was accompanied by an irreversible oxidation of mitochondrial NAD(P)H. When mitochondria were preincubated with CSA, MIBG, ADP or Mg^{2+} before the addition of $3,5(Me)_2$NAPQI, NAD(P)H oxidation was fully reversible (Fig. 4). By contrast, when ADP (data not shown) or CSA was added during Ca^{2+} release, a time-dependent decrease of the extent of $NAD(P)^+$ re-reduction occurred (Fig. 5). HPLC analysis of the incubation medium revealed a time-dependent appearance of NAD^+ and $NADP^+$ in the extramitochondrial medium (Table 1). Only 40–50% of total NAD^+ and $NADP^+$ were detectable in the extramitochondrial medium at a time of near complete Ca^{2+} release (95% of total Ca^{2+} load). When mitochondria were preincubated with CSA before the addition of $3,5(Me)_2$NAPQI, the release of NAD^+ and $NADP^+$ into the medium was prevented (data not shown). Thus, the levels of pyridine nucleotides retained within the mitochondria, as assessed by HPLC analysis (Table 1), closely corresponded to the degree of $NAD(P)^+$ re-reduction observed by spectrophotometric analysis (Fig. 5).

For the measurements of extramitochondrial $NAD(P)^+$ by HPLC, the separation of the mitochondria from the incubation medium by filtration under vacuum was required. To exclude the possibility that the filtration process might have ruptured some of the mitochondria, thereby causing a release of nucleotides into the filtrate, we also tested the extramitochondrial presence of $NADP^+$ using a non-disruptive enzymatic method. The addition of glucose-6-phosphate dehydrogenase together with glucose-6-phosphate to Ca^{2+}-loaded control mitochondria did not alter the absorbance at 375–340 nm (data not shown). However, when enzyme plus substrate was added to mitochondria undergoing $3,5(Me)_2$NAPQI-induced Ca^{2+} release and pyridine nucleotide oxidation, an increase in the absorbance at 340–375 nm was observed (Fig. 6). Thus, mitochondrial $NADP^+$ was accessible to externally added enzymes, confirming the data obtained by HPLC analysis of the filtrate. Estimation of extramitochondrial NAD^+ by enzymatic (sorbitol dehydrogenase catalyzed) reduction proved difficult due to interference by an endogenous mitochondrial NADH oxidizing activity (data not shown).

In addition to pyridine nucleotides, adenine nucleotides were also present in the mitochondrial filtrate (Table 1). The relative amount of individual nucleotides detected in the filtrate varied to a considerable degree, and was highest for AMP, intermediate for ADP and lowest for ATP (Table 1) (71%, 44% and 15%, respectively, at a time when 95% of total Ca^{2+} was released).

Taken together our results show that the Ca^{2+} release pathway was activated in a reversible manner by $3,5(Me)_2$NAPQI and that Ca^{2+} release was accompanied by the release of adenine and pyridine nucleotides from mitochondria. Release of all the nucleotides was prevented by CSA and is therefore concluded to occur through the mitochondrial pore described by Crompton's group [6–11]. It should be noted that despite pore opening, extramitochondrial ATP levels remained low. This explains why mitochondrial ATP was recently found inaccessible to exogenously added hexokinase after NAPQI-mediated Ca^{2+} release was near complete [24]. Conse-

Table 1. Loss of nucleotides from mitochondria during $3,5(Me)_2$NAPQI-induced Ca^{2+} release

Time after addition of $3,5(Me)_2$NAPQI min		Ca^{2+}	ATP	ADP	AMP	NAD^+	$NADP^+$
			nmol/mg protein (% of total)				
0	total		4.03	2.89	3.45	0.93	0.25
	released	(0)	1.05 (26)	0.42 (15)	0.51 (15)	<0.2	<0.2
2	total		5.15	2.73	3.78	4.28	5.84
	released	(95)	0.66 (13)	1.20 (44)	2.68 (71)	2.04 (48)	2.54 (43)
7	total		2.78	2.69	3.75	3.87	4.25
	released	(100)	0.61 (22)	0.94 (35)	2.78 (74)	2.25 (58)	3.19 (75)

Mitochondria were preincubated with succinate (5 mM) and rotenone (3 μM) for 2.5 min, then loaded with Ca^{2+} (15 nmol/mg protein) for 1.5 min before the addition of $3,5(Me)_2$NAPQI (90 nmol/mg protein) (zero time). Duplicate samples were taken at the indicated time points and analysed for total and released nucleotides by HPLC as described in Materials and Methods. Mitochondrial Ca^{2+} release was measured using Arsenazo III. Values are means of duplicate experiments conducted on separate mitochondrial preparations.

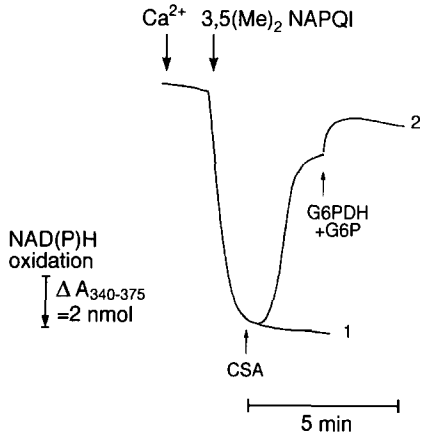

Fig. 6. Presence of oxidized pyridine nucleotides in the extramitochondrial medium. Mitochondria (2 mg/mL) were loaded with Ca^{2+} (15 nmol/mg) as described under Materials and Methods, and exposed to $3,5(Me)_2$NAPQI (90 nmol/mg protein) (trace 1). For trace 2, CSA (1.5 nmol/mg protein) was added 1 min after $3,5(Me)_2$NAPQI and intramitochondrial re-reduction of $NAD(P)^+$ was allowed to proceed to completeness. Subsequently, glucose-6-phosphate (G6P, 250 μM) plus glucose-6-phosphate dehydrogenase (G6PDH, 0.4 U/mL) were added to the mitochondrial incubation as indicated by the arrow. Traces are representative of three independent experiments conducted on separate mitochondrial preparations.

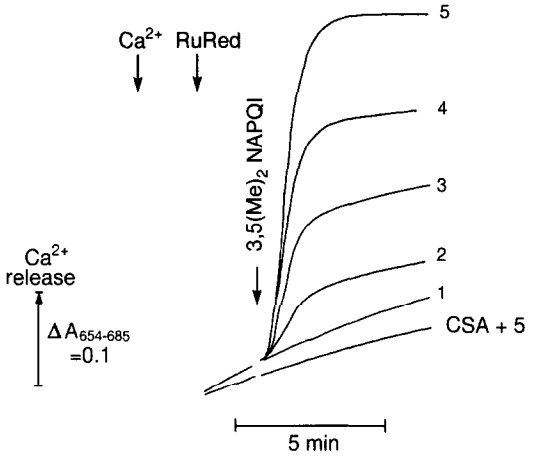

Fig. 7. Concentration-dependence of $3,5(Me)_2$NAPQI-induced mitochondrial Ca^{2+} release in the presence of ruthenium red. Ca^{2+}-loaded mitochondria were incubated for 2 min in the presence of ruthenium red (2 nmol/mg protein) before $3,5(Me)_2$NAPQI was added to the incubation, and Ca^{2+} release measured as described in Materials and Methods. The concentrations of $3,5(Me)_2$NAPQI (nmol/mg protein) were 90 (trace 2), 97 (trace 3), 104 (trace 4), and 110 (trace 5), where trace 5 represents near complete release of all the sequestered Ca^{2+}. Where indicated, 110 nmol/mg protein of $3,5(Me)_2$NAPQI was added to mitochondria preincubated with CSA (1.5 nmol/mg protein) before addition of ruthenium red (trace CSA + 5). Trace 1 is in the absence of any $3,5(Me)_2$NAPQI. Traces are representative of three independent experiments conducted on separate mitochondrial preparations.

quently, extramitochondrial ATP is not an adequate parameter for the estimation of mitochondrial pore formation.

Ca^{2+} release induced by $3,5(Me)_2$NAPQI in the presence of ruthenium red

The addition of ruthenium red, an inhibitor of the Ca^{2+} uniporter [26–28] to Ca^{2+}-loaded control mitochondria produced a slow release of the accumulated Ca^{2+} (basal, ruthenium red-induced release). The subsequent exposure of the mitochondria to a concentration of $3,5(Me)_2$NAPQI (90 nmol/mg protein) that induced rapid release of Ca^{2+} in the absence of ruthenium red (cf. Fig. 1) only slightly enhanced the rate of the basal, ruthenium red-induced Ca^{2+} release (Fig. 7). When increasing the concentration of $3,5(Me)_2$NAPQI (97, 104 or 110 nmol/mg protein), mitochondria released an

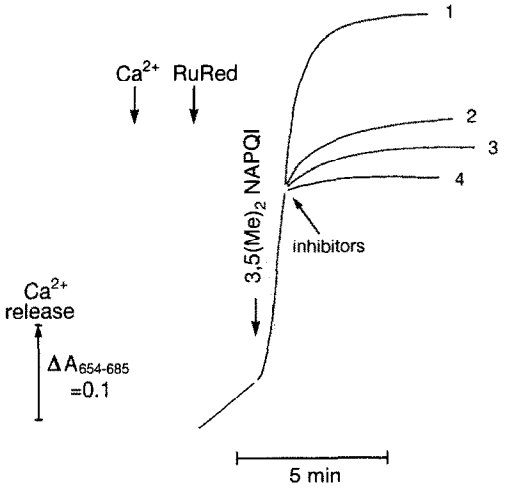

Fig. 8. Effect of CSA, ADP or MIBG on $3,5(Me)_2$NAPQI-induced mitochondrial Ca^{2+} release in the presence of ruthenium red. Ca^{2+}-loaded mitochondria were incubated for 2 min in the presence of ruthenium red (2 nmol/mg protein) and release of all sequestered Ca^{2+} was induced by addition of $3,5(Me)_2$NAPQI (110 nmol/mg protein) (trace 1). Where indicated, MIBG (60 nmol/mg protein) (trace 2), ADP (125 nmol/mg protein) (trace 3), or CSA (1.5 nmol/mg protein) (trace 4) was added during Ca^{2+} release. Traces are representative of three independent experiments conducted on separate mitochondrial preparations.

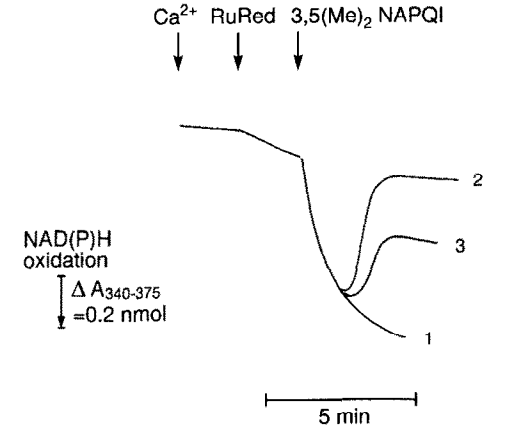

Fig. 9. $3,5(Me)_2$NAPQI-induced pyridine nucleotide oxidation in the presence of ruthenium red. Ca^{2+}-loaded mitochondria were incubated for 2 min in the absence (trace 1) or presence of ruthenium red (2 nmol/mg protein) (traces 2 and 3) before $3,5(Me)_2$NAPQI (90 nmol/mg protein (traces 1 and 2) or 100 nmol/mg protein (trace 3)) was added to the incubation. Traces are representative of three independent experiments conducted on separate mitochondrial preparations.

increasing fraction of the total Ca^{2+}, after which the rate of Ca^{2+} release returned to the basal, ruthenium red-induced rate (Fig. 7). The addition of CSA (Fig. 7) or Mg^{2+} (data not shown) before a high concentration of $3,5(Me)_2$NAPQI (110 nmol/mg protein) inhibited the prooxidant-stimulated Ca^{2+} release also in the presence of ruthenium red. Addition of CSA, MIBG or ADP, but not Mg^{2+} during $3,5(Me)_2$NAPQI-induced Ca^{2+} release immediately prevented any further Ca^{2+} release (Fig. 8).

Mitochondrial NAD(P)H oxidation and nucleotide release in the presence of ruthenium red

As was observed in the absence of ruthenium red, $3,5(Me)_2$NAPQI-induced Ca^{2+} release in the presence of ruthenium red was associated with oxidation of NAD(P)H. NAD(P)H oxidation was either partially reversible or irreversible, depending on whether the concentration of $3,5(Me)_2$NAPQI used caused partial or near complete release of sequestered Ca^{2+} (Fig. 9). HPLC analysis of the filtrate revealed that also in the presence of ruthenium red, Ca^{2+} release was followed by a time-dependent release of adenine and pyridine nucleotides into the extramitochondrial medium (Table 2). Recovery of nucleotides in the filtrate varied for individual nucleotides and was highest for AMP, lowest for ATP and intermediate for ADP, NAD^+ and $NADP^+$ (Table 2). This pattern was similar to that observed in the absence of ruthenium red. Consequently, $3,5(Me)_2$NAPQI-induced mitochondrial Ca^{2+} release was followed by pore opening also in the presence of ruthenium red.

Role of extramitochondrial versus intramitochondrial Ca^{2+} in the induction of Ca^{2+} release

Ruthenium red may not completely block Ca^{2+} uptake [20], leaving open the possibility of a limited degree of Ca^{2+}-cycling. Therefore, the possibility of minimal Ca^{2+}-cycling contributing to pore opening was tested by increasing the extramitochondrial Ca^{2+} concentration. To this aim, an additional 7.5 nmol of Ca^{2+}/mg mitochondrial protein were added to Ca^{2+}-loaded mitochondria (15 nmol Ca^{2+}/mg protein) after blocking the uniporter with ruthenium red. The increased extramitochondrial Ca^{2+} concentration did not affect Ca^{2+} release induced by $3,5(Me)_2$NAPQI (97 nmol/mg protein, a concentration which caused release of about 50% of the sequestered Ca^{2+}) (Fig. 10). Instead, when the same amount of Ca^{2+} (i.e. 7.5 nmol/mg protein) was added and allowed to be completely sequestered by mitochondria (preloaded with 15 nmol Ca^{2+}/mg protein) before the addition of ruthenium red, 97 nmol of $3,5(Me)_2$NAPQI/mg protein induced near complete release of all the sequestered Ca^{2+} (i.e. 22.5 nmol Ca^{2+}/mg protein) (Fig. 10). Again, Ca^{2+} release was prevented and NAD(P)H oxidation was reversible on preincubation with CSA (data not shown).

Effect of CSA, ADP or MIBG on the basal, ruthenium red-induced Ca^{2+} release

As shown in Fig. 11, CSA, MIBG and ADP also decreased the rate of the basal, ruthenium red-

Table 2. Loss of nucleotides from mitochondria during $3,5(Me)_2$NAPQI-induced Ca^{2+} release in the presence of ruthenium red

Time after addition of $3,5(Me)_2$NAPQI min		Ca^{2+}	ATP	ADP	AMP	NAD^+	$NADP^+$
			nmol/mg protein (% of total)				
0	total		5.53	2.66	3.92	0.35	<0.2
	released	(0)	1.05 (19)	0.40 (15)	0.41 (11)	<0.2	<0.2
2	total		4.74	3.43	3.99	3.50	4.38
	released	(95)	0.79 (17)	1.25 (36)	1.86 (47)	1.86 (53)	1.50 (34)
7	total		4.75	2.57	3.35	3.58	3.51
	released	(100)	0.92 (19)	1.25 (49)	2.27 (68)	2.16 (58)	2.50 (60)

Mitochondria were preincubated with succinate (5 mM) and rotenone (3 μM) for 2.5 min, then loaded with Ca^{2+} (15 nmol/mg protein) for 2 min before the addition of ruthenium red (2 nmol/mg protein). After an additional 2 min, $3,5(Me)_2$NAPQI (110 nmol/mg protein) was added (zero time). Duplicate samples were taken at the indicated time points and analyzed for total and released nucleotides by HPLC as described in Materials and Methods. Mitochondrial Ca^{2+} release was measured using arsenazo III. Values are means of duplicate experiments conducted on separate mitochondrial preparations.

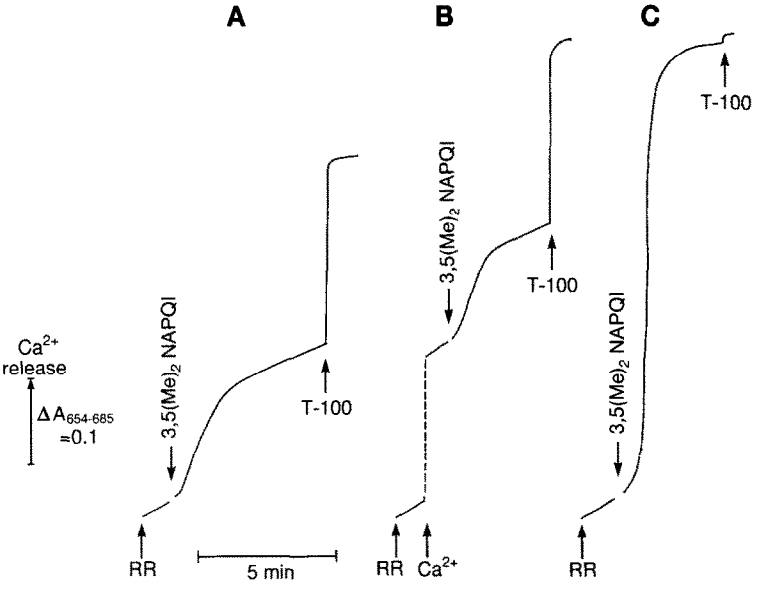

Fig. 10. Effect of extra- versus intramitochondrial Ca^{2+} on $3,5(Me)_2$NAPQI-induced Ca^{2+} release in the presence of ruthenium red. Ca^{2+}-loaded mitochondria were incubated for 2 min in the presence of ruthenium red (2 nmol/mg protein) before $3,5(Me)_2$NAPQI (97 nmol/mg protein) was added to the incubation medium (A). In (B) and (C) an additional 7.5 nmol of Ca^{2+}/mg protein were added to the incubation after (B) or before (C) uptake was inhibited by ruthenium red. Traces are representative of three independent experiments conducted on separate mitochondrial preparations. Abbreviation used: T-100, Triton X-100.

induced Ca^{2+} release by around 45% (ADP, MIBG) to 70% (CSA); however, neither of these reagents could prevent its release completely. The results suggest that the basal, ruthenium red-induced mitochondrial Ca^{2+} release involves at least two components, one of which is sensitive to the same agents that inhibit the prooxidant-stimulated Ca^{2+} release.

DISCUSSION

In this study we have shown that opening of the Ca^{2+}-dependent mitochondrial pore [6–11] occurred during the stimulation of Ca^{2+} release from mitochondria by the prooxidant $3,5(Me)_2$NAPQI. This was evidenced by (i) the release of mitochondrial adenine and pyridine nucleotides and (ii) the reversibility of opening induced by ADP and CSA, in agreement with other reports [29, 30]. In addition to CSA or ADP, MIBG and Mg^{2+} were found to be inhibitors of both Ca^{2+} release and pore opening.

In the absence of ruthenium red, Ca^{2+} release markedly preceded the appearance of mitochondrial nucleotides in the extramitochondrial medium. This

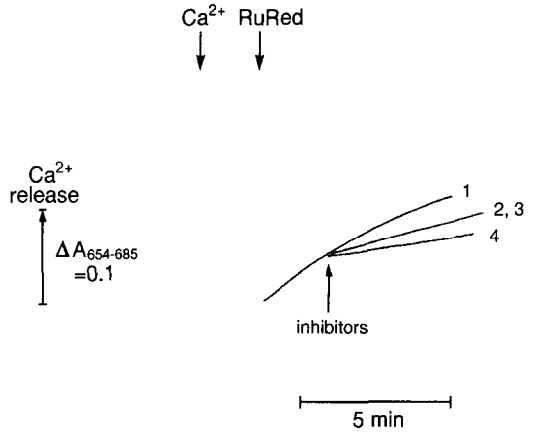

Fig. 11. Effect of CSA, ADP or MIBG on the basal, ruthenium red-induced mitochondrial Ca^{2+} release. Ca^{2+}-loaded mitochondria were incubated for 2 min in the presence of ruthenium red (2 nmol/mg protein) (trace 1). Where indicated, MIBG (60 nmol/mg protein) (trace 2), ADP (150 nmol/mg protein) (trace 3), or CSA (1.5 nmol/mg protein) (trace 4) was added. Trace 1 is in the absence of any inhibitor. Traces are representative of three independent experiments conducted on separate mitochondrial preparations.

difference in kinetics could be due to different rates of release through the mitochondrial pore [8] or, alternatively, activation of an additional Ca^{2+}-selective pathway by the prooxidant. Indeed, the existence of such a Ca^{2+}-selective pathway that can be activated by prooxidants has been suggested by many reports [31–37], although the participation of the Ca^{2+}-dependent pore could not be ruled out unequivocally. However, Schlegel *et al.* have recently shown that tBuOOH activates a Ca^{2+}-selective release pathway without pore opening under experimental conditions where Ca^{2+} reuptake through the uniporter was prevented by ruthenium red or EGTA [20]. In agreement with that report, pore opening was not induced in the presence of ruthenium red when using a concentration of $3,5(Me)_2$NAPQI that caused pore opening in the absence of ruthenium red. However, in contrast with tBuOOH, Ca^{2+} release was only minimally stimulated by this concentration of $3,5(Me)_2$NAPQI when ruthenium red was present. When the concentration of the prooxidant was increased sufficiently to cause release of Ca^{2+} in the presence of ruthenium red, nucleotides were also lost from the mitochondria. As was observed in the absence of ruthenium red, ADP, CSA, Mg^{2+} and MIBG were inhibitory on both Ca^{2+} and nucleotide release. Thus, in the case of the prooxidant $3,5(Me)_2$NAPQI, blocking the uniporter by ruthenium red did not allow a clear dissociation between a Ca^{2+}-selective efflux pathway and mitochondrial pore opening.

Ruthenium red may not completely block Ca^{2+} uptake [20], leaving open the possibility of a limited degree of Ca^{2+}-cycling. If this low level of Ca^{2+}-cycling were the critical event triggering pore opening by $3,5(Me)_2$NAPQI in the presence of ruthenium red, increasing the extramitochondrial Ca^{2+} concentration would be expected to stimulate $3,5(Me)_2$NAPQI-induced pore opening and Ca^{2+} release. However, this was not the case. On the other hand, an increase in the intramitochondrial Ca^{2+} load was found to promote the Ca^{2+} release. Consequently, the results from this study do not support a role for the process of Ca^{2+}-cycling in pore opening by $3,5(Me)_2$NAPQI, but implicate the intramitochondrial Ca^{2+} load as a critical factor in triggering pore opening.

In the presence of ruthenium red, mitochondria released a concentration-dependent fraction of all sequestered Ca^{2+} upon addition of $3,5(Me)_2$NAPQI. The latter finding clearly shows that the mitochondrial suspension does not react homogeneously to $3,5(Me)_2$NAPQI, but rather that mitochondrial subpopulations of different sensitivity of Ca^{2+} release towards the concentration of prooxidant exist, as originally suggested from tBuOOH-induced mitochondrial swelling experiments [38].

The existence of subpopulations with different sensitivities towards $3,5(Me)_2$NAPQI may explain why different concentrations of $3,5(Me)_2$NAPQI are required to induce near complete Ca^{2+} release depending on the absence versus presence of ruthenium red. Thus, only a limited fraction of mitochondria releases the sequestered Ca^{2+} upon addition of 90 nmol $3,5(Me)_2$NAPQI/mg protein. In the presence of ruthenium red Ca^{2+} remains extramitochondrially; however, in the absence of ruthenium red Ca^{2+} is taken up by a fraction that is less sensitive to the prooxidant. The increased mitochondrial Ca^{2+} load in turn lowers the sensitivity of these mitochondria towards the prooxidant, thereby leading to a cascade of further, and eventually complete, Ca^{2+} release.

Taken together the results obtained in the absence versus the presence of ruthenium red allow the following conclusions about the critical factors regulating $3,5(Me)_2$NAPQI-induced mitochondrial Ca^{2+} release to be drawn. $3,5(Me)_2$NAPQI-induced mitochondrial Ca^{2+} release is a cooperative phenomenon, which is regulated by at least two different parameters. These are (i) the prooxidant concentration, which acts synergistically with (ii) the mitochondrial Ca^{2+} load. The open probability of the mitochondrial permeability transition pore (MTP) measured in swelling experiments using depolarized mitochondria was similarly reported to be regulated by the synergistic action of (i) matrix alkalinization, and (ii) the mitochondrial Ca^{2+} content, with matrix acidification being antagonized by the Ca^{2+} content [18]. It is challenging to speculate that the synergism observed here between the Ca^{2+} load and the prooxidant concentration relate to the synergism observed between Ca^{2+} load and mitochondrial pH. The prooxidant concentration would be reflected in the matrix pH through the mitochondrial NAD(P)H redox state according to the following equation:

$$NAD(P)H + H^+ + 3,5(Me)_2NAPQI \leftrightharpoons NAD(P)^+ + 3,5(Me)_2NAPQIH_2,$$

where protons are consumed during the reaction. This suggestion is supported by the fact that the

chemical oxidation of NADH by 3,5(Me)$_2$NAPQI (25 μM) is accompanied by an increase in pH of more than 0.4 pH units [M. Weis, unpublished observation].

The agents shown here to inhibit the prooxidant-induced mitochondrial Ca^{2+} release also inhibited the basal, ruthenium red-induced Ca^{2+} release by around 45–70%, suggesting that the basal ruthenium red-induced Ca^{2+} release involves, in part, the same pathway. The preventive action of CSA, ADP and Mg^{2+} on mitochondrial Ca^{2+} release is likely to relate to the interaction of the compounds with the mitochondrial pore complex. The mechanism of action underlying the protective action of MIBG is less clear. Thus, MIBG is an inhibitor of protein mono(ADP)ribosylation [39]; however, its instantaneous action in preventing further Ca^{2+} release and pore opening when added after the prooxidant (in the presence of ruthenium red) as well as its increased protective effect at higher mitochondrial Ca^{2+} load [M. Weis, unpublished observations] suggests the possibility of an additional mode of action, i.e. competition with Ca^{2+} for the Ca^{2+} binding site regulating pore opening.

Little is known about the possible physiological role of the mitochondrial pore. Since pore opening was completely prevented at a concentration of Mg^{2+} that was much lower than that found in the cytosol of mammalian cells [40], pore opening would be expected to be restricted to pathological rather than physiological conditions of the cell. However, we have recently reported that CSA [4] and MIBG [41] lead to a rapid accumulation of Ca^{2+} by mitochondria in isolated hepatocytes. Whether the latter observation reflects a low level of pore opening under physiological conditions or an additional Ca^{2+} release pathway that is sensitive to CSA and MIBG, as suggested by the work of Schlegel *et al.* [20], remains unclear.

In summary, the present data show that the ruthenium red-induced and the 3,5(Me)$_2$NAPQI-induced mitochondrial Ca^{2+} release occur through a common component. Considering the well known role of several of these inhibitors in regulating the mitochondrial pore, it is proposed that the component mediating prooxidant-induced and ruthenium red-induced Ca^{2+} release is involved in mitochondrial pore formation.

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