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PHENYLARSINE OXIDE STIMULATES PYRIDINE NUCLEOTIDE-LINKED Ca^{2+} RELEASE FROM RAT LIVER MITOCHONDRIA

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Abstract—Rat liver mitochondria contain a specific Ca^{2+} release pathway which operates when oxidized mitochondrial pyridine nucleotides are hydrolysed to ADPribose and nicotinamide. Here we report that the hydrophobic bifunctional thiol reagent phenylarsine oxide (PhAsO) at low concentrations stimulates this pathway by promoting a Ca^{2+}-dependent hydrolysis of oxidized mitochondrial pyridine nucleotides. Ca^{2+} release is inhibited by cyclosporine A or *m*-iodobenzylguanidine, compounds known to prevent intramitochondrial pyridine nucleotide hydrolysis or protein mono(ADPribosyl)ation, respectively. At higher concentrations, PhAsO causes non-specific leakiness of mitochondria.

Key words: calcium release; intactness; membrane potential; pyridine nucleotides; sulfhydryls

Uptake and release of mitochondrial Ca^{2+} occur via different pathways [1]. Lehninger and co-workers were the first to report that oxidation of mitochondrial pyridine nucleotides stimulates Ca^{2+} release [2]. We subsequently showed that pyridine nucleotide oxidation is necessary but not sufficient for Ca^{2+} release; rather, it occurs when oxidized pyridine nucleotides in Ca^{2+}-loaded mitochondria are hydrolysed at the bond between the ADPribose and nicotinamide moiety. We also provided evidence that subsequent to pyridine nucleotide hydrolysis protein mono(ADPribosyl)ation triggers Ca^{2+} release (reviewed in [3, 4]). Thus, a protein in the inner mitochondrial membrane serves as an acceptor for monoADPribose [5], and Ca^{2+} release is accompanied by reversible protein mono(ADP-ribosyl)ation in mitochondria [6]. Accordingly, inhibitors which prevent pyridine nucleotide hydrolysis or protein mono(ADPribosyl)ation, such as CSA† [7, 8] or MIBG [9], also prevent Ca^{2+} release.

We now report that the hydrophobic bifunctional thiol reagent phenylarsine oxide (PhAsO) stimulates Ca^{2+} release from rat liver mitochondria. PhAsO-promoted Ca^{2+} release can occur with preservation of $\Delta\Psi$ and without mitochondrial swelling. Release is accompanied by Ca^{2+}-dependent pyridine nucleotide hydrolysis and is inhibited by CSA or MIBG. PhAsO does not oxidize reduced pyridine nucleotides. We conclude that at low PhAsO concentrations Ca^{2+} release is triggered by a PhAsO-induced, Ca^{2+}-dependent hydrolysis of oxidized pyridine nucleotides and occurs from intact mitochondria. Higher PhAsO concentrations, however, cause non-specific leakiness of mitochondria.

MATERIALS AND METHODS

Materials. CSA was a gift from Sandoz Pharma Preclinical Research (Basel, Switzerland). It was stored in solid form at −20° and dissolved in ethanol immediately prior to use. All other chemicals were purchased from standard suppliers, and were of the highest purity commercially available. PhAsO was dissolved in ethanol/50 mM LiOH.

Isolation of mitochondria. The isolation of rat liver mitochondria was performed by differential centrifugation as described [10]. Protein content was determined by the Biuret method with BSA as standard.

Labelling of mitochondrial pyridine nucleotides in vivo. Overnight-fasted rats were injected i.v. with [*carboxyl*-^{14}C]nicotinic acid (12.5 μCi, 0.223 μmol) [11] in phosphate-buffered saline. After 3 hr, the animals were killed and liver mitochondria isolated.

Standard incubation procedure. Mitochondria (2 mg of protein/mL) were incubated at 25° with continuous stirring and oxygenation in 3 mL MSH buffer.

Determination of Ca^{2+} *uptake and release by mitochondria.* The standard incubation procedure was followed. After addition of rotenone (5 μM) and potassium succinate (2.5 mM) mitochondria were loaded with Ca^{2+}. Its pathway across the inner

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† Abbreviations: CSA, cyclosporine A; $\Delta\Psi$, electrical potential across the inner mitochondrial membrane, negative inside; FCCP, *p*-trifluoromethoxyphenylhydrazone; MIBG, *m*-iodobenzylguanidine; MSH buffer, 210 mM mannitol, 70 mM sucrose, 5 mM HEPES, pH 7.2; PhAsO, phenylarsine oxide; tbh, *t*-butylhydroperoxide.

mitochondrial membrane was monitored by the spectrophotometric or isotope technique (Millipore filtration) as described [10, 11]. 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 figures, and its uptake allowed to proceed for 2 min. Finally, PhAsO (in the spectrophotometric determination) or 5 mM EGTA alone or in combination with PhAsO (in the isotope technique measurement) was added at time 0 min as indicated in the figures.

Determination of mitochondrial membrane potential. Mitochondria were incubated according to the standard procedure in the presence of 10 μM safranine. After addition of rotenone (5 μM) and potassium 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 [12].

Measurement of mitochondrial swelling. Swelling of mitochondria incubated according to the standard procedure was monitored continuously as changes in OD_{540}.

Spectrophotometric analysis of mitochondrial pyridine nucleotides. The standard incubation procedure was followed. After addition of rotenone (5 μM) and potassium succinate (2.5 mM) the absorption of mitochondrial pyridine nucleotides was determined in an Aminco DW2A spectrophotometer at 340–370 nm [8]. Other compounds were added as indicated in the figures.

Determination of intramitochondrial pyridine nucleotide hydrolysis. Rats were injected i.v. 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) (except for Fig. 4C) and potassium succinate (2.5 mM), and at time 0 min PhAsO alone or in combination with the indicated compounds was added. Release of nicotinamide as an indicator of pyridine nucleotide hydrolysis [11] was followed with the Millipore filtration technique [10].

RESULTS

Stimulation by phenylarsine oxide of Ca^{2+} *release*

PhAsO stimulates Ca^{2+} release from mitochondria as shown by the spectrophotometric and the isotope technique (Fig. 1). From Fig. 1A it is evident that with a given Ca^{2+} load the time of onset of Ca^{2+} release depends on the amount of PhAsO offered, and Fig. 1B shows that with a given amount of PhAsO the kinetics of the PhAsO-dependent Ca^{2+}

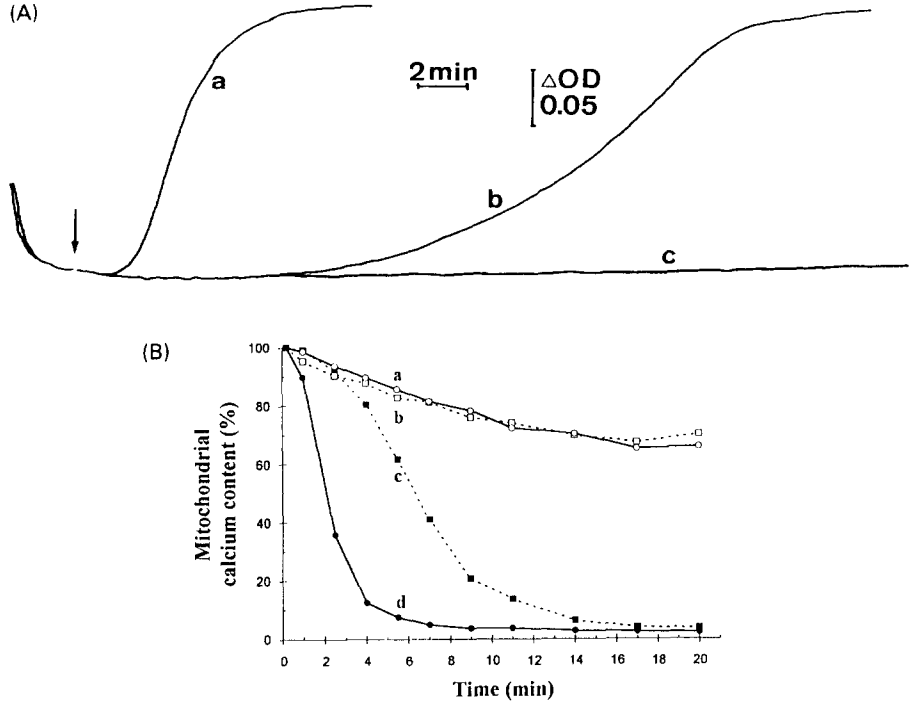

Fig. 1. Phenylarsine oxide-stimulated Ca^{2+} release from rat liver mitochondria. Mitochondria were incubated according to the standard procedure. (A) Spectrophotometric assay. After addition of rotenone and potassium succinate mitochondria were loaded with 40 nmol of Ca^{2+}/mg of protein. At the *arrow*, 10 μM (trace a) or 3.5 μM (trace b) PhAsO was added to stimulate Ca^{2+} release. Trace c: Vehicle alone. (B) Isotope technique. After addition of rotenone and potassium succinate mitochondria were loaded with Ca^{2+}, and 2 min thereafter (time 0 min) 5 mM EGTA was added either alone or in combination with PhAsO. Trace a, 40 nmol Ca^{2+}/mg of protein; trace b, 20 nmol Ca^{2+}/mg of protein; trace c, 20 nmol Ca^{2+}/mg of protein, 3.5 μM PhAsO; trace d, 40 nmol Ca^{2+}/mg of protein, 3.5 μM PhAsO. The results shown are from one experiment typical of four.

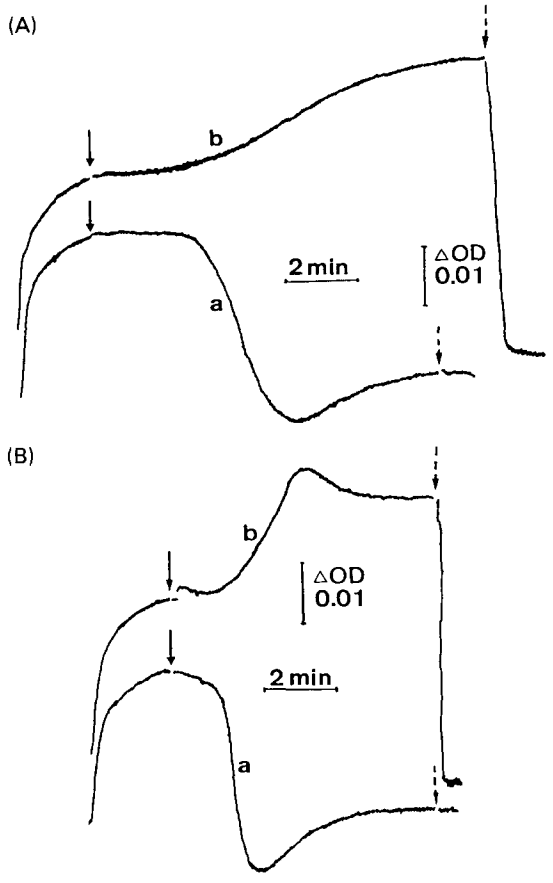

Fig. 2. Phenylarsine oxide-induced alterations of mitochondrial membrane potential. Mitochondria were incubated according to the standard procedure in the presence of 10 μM safranine and energized in the presence of rotenone with potassium succinate. Two minutes after addition of Ca^{2+} (20 nmol/mg of protein) PhAsO alone (traces a) or together with 5 mM EGTA (traces b) was added (solid *arrow*). At the dashed *arrow* 1 μM FCCP was added. (A) 3.5 μM PhAsO, (B) 10 μM PhAsO. The results shown are from one experiment typical of four.

release depend on the amount of Ca^{2+} previously accumulated by mitochondria.

Intactness of mitochondria during phenylarsine oxide-induced Ca^{2+} release

The determination of $\Delta\Psi$ is useful in assessing the intactness of mitochondria during and after Ca^{2+} release [13]. When PhAsO is added to mitochondria loaded with Ca^{2+} (20 nmol/mg of protein) $\Delta\Psi$ changes depend on whether or not mitochondria are allowed to cycle (continuously release and take up) Ca^{2+} (Fig. 2). Thus, $\Delta\Psi$ soon decreases after addition of PhAsO in the absence of EGTA (traces a), but increases in its presence (traces b); i.e. under conditions which prevent Ca^{2+} cycling. Accordingly, collapse of $\Delta\Psi$ is now achieved by the addition of the uncoupler FCCP. Mitochondrial swelling was monitored as another indicator of non-specific permeability changes of the inner mitochondrial membrane. When 3.5 μM PhAsO was added to Ca^{2+}-loaded mitochondria (20 or 40 nmol of Ca^{2+}/mg of protein) (Fig. 3A), they swell in the absence (traces c and d) but not in the presence (traces a and b) of EGTA. With 10 μM PhAsO, substantial swelling is observed even in the presence of EGTA (Fig. 3B).

Fate of mitochondrial pyridine nucleotides

Intact rat liver mitochondria release Ca^{2+} via a specific pathway when their pyridine nucleotides are hydrolysed to ADPribose and nicotinamide. It was therefore of interest to investigate whether this pathway is stimulated by PhAsO. Figure 4A reports the spectrophotometric analysis of mitochondrial pyridine nucleotides. In Ca^{2+}-depleted mitochondria PhAsO does not influence pyridine nucleotides (trace a). However, PhAsO evokes a drastic decrease of their absorption at 340–370 nm in Ca^{2+}-loaded mitochondria in the absence (trace b) but not in the presence (trace c) of 1 μM CSA. The direct determination of pyridine nucleotide hydrolysis, assayed by nicotinamide release from mitochondria

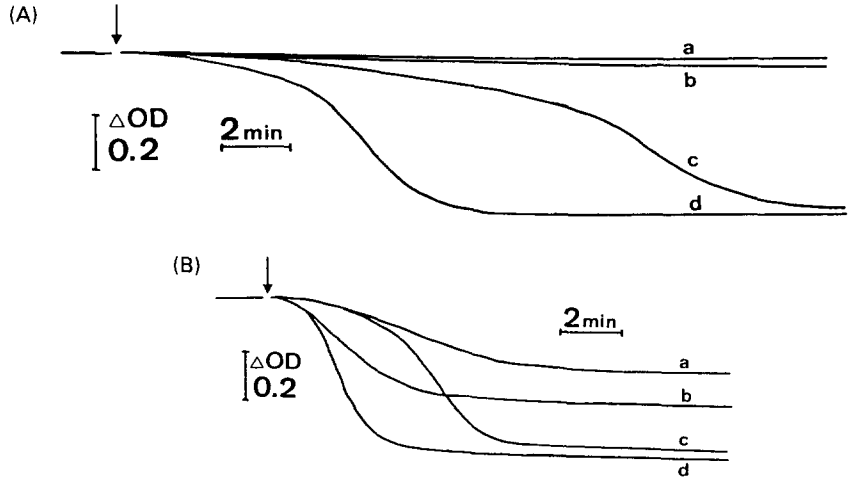

Fig. 3. Phenylarsine oxide-induced swelling of mitochondria. Mitochondria were incubated according to the standard procedure and energized with succinate in the presence of rotenone. Two minutes after addition of Ca^{2+} (traces a and c, 20 nmol/mg of protein; traces b and d, 40 nmol/mg of protein) PhAsO alone (traces c and d) or in combination with 10 mM EGTA (traces a and b) was added (*arrow*). (A) 3.5 μM PhAsO, (B) 10 μM PhAsO. The results shown are from one experiment typical of three.

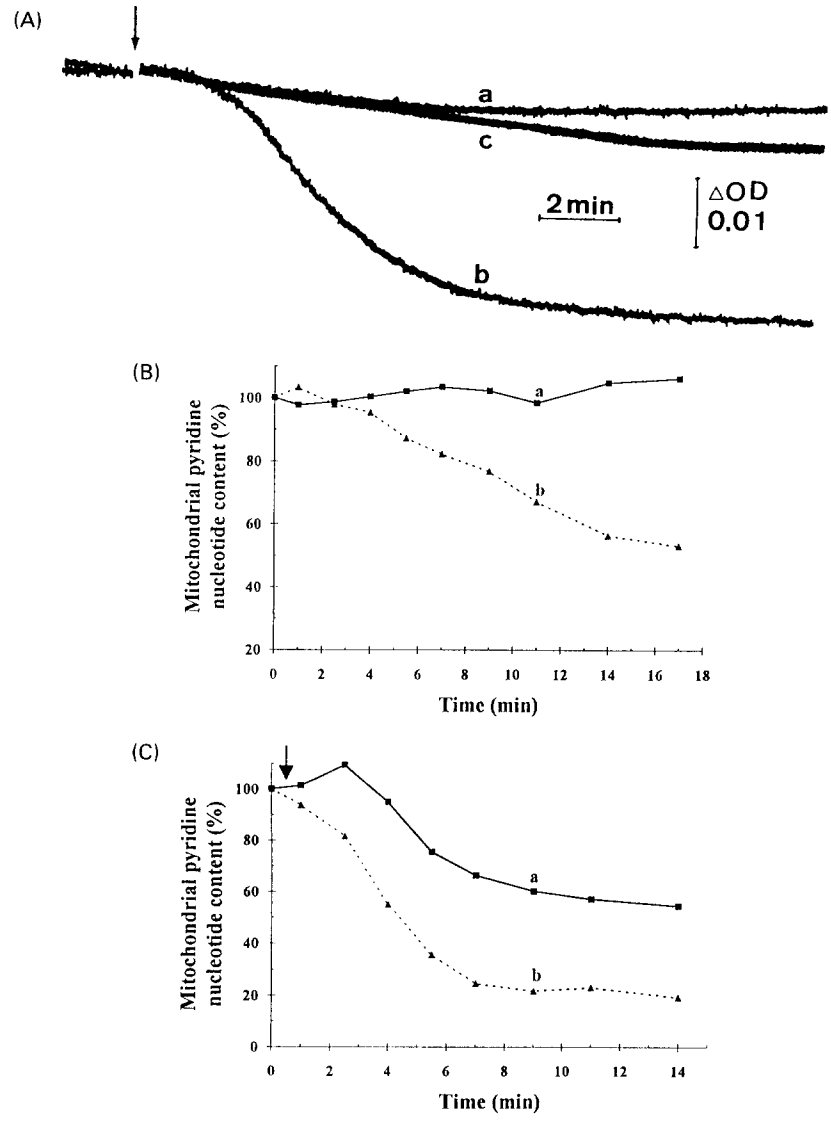

Fig. 4. Phenylarsine oxide-induced alterations of mitochondrial pyridine nucleotides. (A) Spectrophotometric analysis at 340–370 nm. Trace a: 2 min after addition of rotenone, 5 mM EGTA, and 30 sec thereafter potassium succinate were added. After another 2 min (*arrow*), 10 μM PhAsO was added. Other traces: mitochondria were incubated according to the standard procedure in the absence (trace b) or presence (trace c) of 1 μM CSA. Two minutes after addition of Ca^{2+} (20 nmol/mg of protein), 10 μM PhAsO together with 5 mM EGTA was added (*arrow*). (B) Mitochondria labelled at the nicotinamide moiety were incubated according to the standard procedure, exposed to rotenone and potassium succinate, and either depleted of Ca^{2+} (trace a, conditions as for trace a in Panel A) or loaded with 30 nmol of Ca^{2+}/mg of protein (trace b). At time 0 min 3.5 μM PhAsO in combination with 5 mM EGTA was added. Hydrolysis of pyridine nucleotides was determined by analysis of nicotinamide release with Millipore filtration. (C) Mitochondria were incubated without rotenone in the presence of EGTA (5 mM). Thereafter, 100 μM tbh was added, followed 30 sec later by potassium succinate. Two minutes later 10 μM PhAsO (trace b) or its vehicle (trace a) was added and after one more min (time 0 min) the analysis of nicotinamide release with Millipore filtration was started. At time 30 sec (*arrow*) 75 nmol of Ca^{2+}/mg of protein was added. The results shown are from one experiment typical of three.

(Fig. 4B and C), reveals that PhAsO stimulates in a Ca^{2+}-dependent manner the hydrolysis of oxidized pyridine nucleotides. Thus, hydrolysis is not observed in Ca^{2+}-depleted mitochondria (Fig. 4B, trace a), but occurs in Ca^{2+}-loaded mitochondria (Fig. 4B, trace b). When pyridine nucleotide hydrolysis is initiated by addition of Ca^{2+} to mitochondria whose pyridine nucleotides are oxidized due to the presence of tbh [14] their hydrolysis is further stimulated by PhAsO (Fig. 4C).

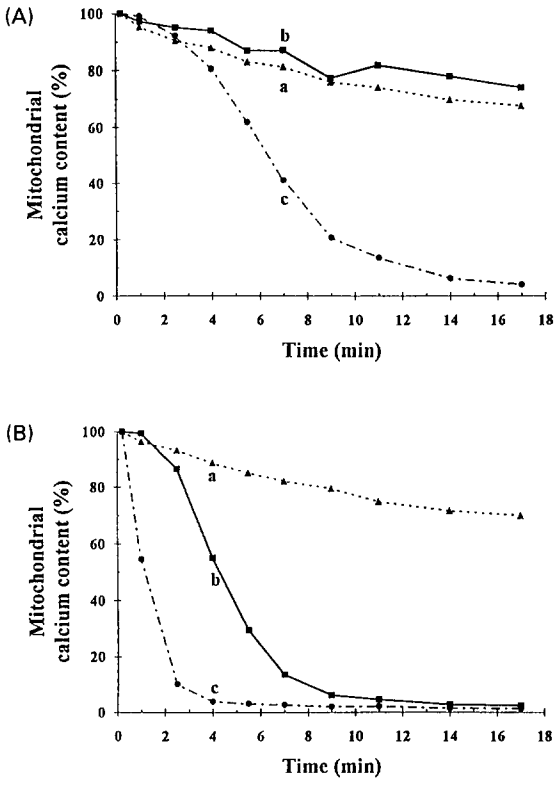

Fig. 5. Inhibition by cyclosporine A of phenylarsine oxide-stimulated Ca^{2+} release from mitochondria. Mitochondria were incubated according to the standard procedure in the presence or absence of 1 μM CSA. After addition of rotenone and potassium succinate they were loaded with 20 (Panel A) or 40 (Panel B) nmol of Ca^{2+}/mg of protein. At time 0 min PhAsO or its vehicle was added. Ca^{2+} release was determined by Millipore filtration. (A) Trace a, vehicle alone; trace b, 3.5 μM PhAsO, 1 μM CSA; trace c, 3.5 μM PhAsO. (B) trace a, vehicle alone; trace b, 10 μM PhAsO, 1 μM CSA; trace c, 10 μM PhAsO. The results shown are from one experiment typical of three.

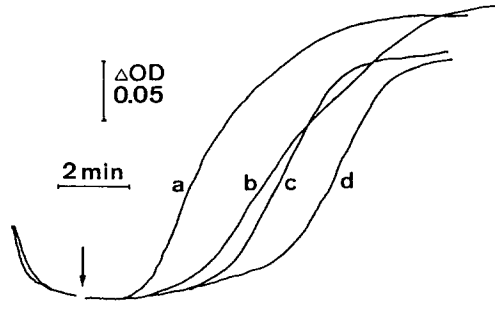

Fig. 6. Inhibition by *m*-iodobenzylguanidine of phenylarsine oxide-stimulated Ca^{2+} release from mitochondria. Mitochondria were incubated according to the standard procedure in the presence or absence of MIBG. After addition of rotenone and potassium succinate they were loaded with 40 nmol of Ca^{2+}/mg of protein. At the *arrow*, PhAsO was added. Trace a, 10 μM PhAsO; trace b, 10 μM PhAsO, 90 μM MIBG; trace c, 5 μM PhAsO; trace d, 5 μM PhAsO, 90 μM MIBG. The results shown are from one experiment typical of three.

Sensitivity of phenylarsine oxide-induced Ca^{2+} *release to cyclosporine A or* m-*iodobenzylguanidine*

Prooxidant-induced, pyridine nucleotide-linked Ca^{2+} release from mitochondria is inhibited by compounds which interfere with protein mono-(ADPribosyl)ation. They also inhibit PhAsO-induced Ca^{2+} release (Figs 5 and 6). Figure 5 shows that CSA completely prevents PhAsO-induced Ca^{2+} release under moderate conditions (20 nmol Ca^{2+}/mg of protein, 3.5 μM PhAsO) (Fig. 5A), and clearly impedes it under more drastic conditions (40 nmol Ca^{2+}/mg of protein, 10 μM PhAsO) (Fig. 5B). Inhibition by CSA of Ca^{2+} release is paralleled by inhibition of pyridine nucleotide oxidation and hydrolysis as judged from nicotinamide release measurements (results not shown). Also MIBG retards PhAsO-induced Ca^{2+} release (Fig. 6), but, however, influences neither pyridine nucleotide oxidation nor nicotinamide release from mitochondria (results not shown).

DISCUSSION

We have recently shown [10] that: (1) pyridine nucleotide-linked Ca^{2+} release from rat liver mitochondria does not require a non-specific permeability transition ("pore" formation) in the inner mitochondrial membrane; (2) pyridine nucleotide-linked Ca^{2+} release occurs *via* a specific pathway from intact mitochondria; and (3) "pore" formation is likely to be secondary to Ca^{2+} cycling by mitochondria.

Protein-SH groups have long been known to be critically involved in the regulation of cation fluxes through the inner mitochondrial membrane [15]. Since PhAsO modifies vicinal thiol groups and promotes the movement of ions, among them Ca^{2+}, through the inner mitochondrial membrane [16–18], we investigated whether PhAsO stimulates specific Ca^{2+} release from mitochondria. We found that when Ca^{2+} cycling is prevented PhAsO at low concentrations (3.5 μM) stimulates Ca^{2+} release from intact mitochondria, as judged by $\Delta\Psi$, swelling, and pyridine nucleotide hydrolysis measurements. However, at 10 μM PhAsO mitochondria are damaged even in the presence of EGTA or cyclosporine A.

PhAsO has been shown to induce a non-specific permeability transition in mitochondria [16, 19] under conditions very different from those used in our study; e.g. at very high PhAsO concentrations (up to 400 μM) and in the presence of phosphate. CSA [16] and butylated hydroxytoluene [16, 18], another inhibitor of mitochondrial pyridine nucleotide hydrolysis [20], also inhibited permeability transition.

In contrast to prooxidants such as tbh [14], alloxan [21], or menadione [22], PhAsO does not directly cause pyridine nucleotide oxidation, but instead stimulates Ca^{2+}-dependent hydrolysis of oxidized pyridine nucleotides. This conclusion is based on two findings. Firstly, PhAsO does not affect reduced pyridine–nucleotides in Ca^{2+}-depleted mitochondria.

In contrast, with Ca^{2+}-loaded mitochondria their absorption at 340–370 nm decreases massively in the presence of PhAsO, but only slightly in the presence of PhAsO *plus* CSA (Fig. 4A). The latter notion is in accordance with the reports that CSA inhibits Ca^{2+}-dependent hydrolysis of oxidized pyridine nucleotides in mitochondria [7, 8]. It should be noted here that between 14 and 43% of pyridine nucleotides are oxidized in Ca^{2+}-loaded mitochondria in the presence of rotenone and succinate ([14], and Frei and Richter, unpublished result). The Ca^{2+} requirement for pyridine nucleotide hydrolysis is confirmed by nicotinamide release measurements (Fig. 4B), which also show that hydrolysis stops when Ca^{2+} release is complete under non-cycling conditions. Secondly, when mitochondrial pyridine nucleotides are extensively oxidized due to reduction of tbh by glutathione peroxidase, their hydrolysis induced by the addition of Ca^{2+} is accelerated by PhAsO (Fig. 4C). The PhAsO-induced disappearance of reduced pyridine nucleotides in Ca^{2+}-containing mitochondria is, therefore, most likely due to PhAsO-stimulated hydrolysis of oxidized pyridine nucleotides followed by oxidation of reduced pyridine nucleotides in response to thermodynamic equilibration.

The exact mode of action of PhAsO in mitochondria is not yet known. Depletion of mitochondrial glutathione by phorone treatment of rats [23] does not alter the response of mitochondria to PhAsO (unpublished result). According to Novgorodov *et al.* [18, 19] the hydrophobic thiol reagent *N*-ethylmaleimide, but not the hydrophilic thiol reagent mersalyl, prevents effects of PhAsO in mitochondria. Although the existence of a NAD^+ glycohydrolase in the inner mitochondrial membrane, first proposed by us [11], is currently under debate [24], we envisage that the highly lipophilic PhAsO activates the protein in the membrane. It is also conceivable that PhAsO inactivates a putative inhibitor of NAD^+ glycohydrolase. We have recently provided evidence that the mitochondrial petidyl-prolyl *cis-trans* isomerase, a target of CSA, regulates mitochondrial pyridine nucleotide hydrolysis and Ca^{2+} release [8]. It is tempting to speculate that this protein is under the inhibitory control of a naturally occuring CSA analogue and is activated by PhAsO.

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