CMLS, Cell. Mol. Life Sci. 59 (2002) 1190–1197
1420-682X/02/071190-08 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Research Article

Peroxovanadate inhibits Ca^{2+} release from mitochondria

M. Salvi[a], A. Toninello[a], M. Schweizer[b,‡], S. D. Friess[c] and C. Richter[b,*]

[a] Department of Biological Chemistry, University of Padova and CNR Center for the Study of Biomembranes, 35121 Padova (Italy)
[b] Institute of Biochemistry, Swiss Federal Institute of Technology (ETH), 8092 Zurich (Switzerland), Fax + 41 1 632 11 21, e-mail: richter@bc.biol.ethz.ch
[c] Laboratory of Organic Chemistry, Swiss Federal Institute of Technology (ETH), 8093 Zurich (Switzerland)

Received 28 March 2002; received after revision 8 May 2002; accepted 15 May 2002

Abstract. Mitochondria contain a specific Ca^{2+} release pathway which operates when oxidized mitochondrial pyridine nucleotides are hydrolyzed. NAD^+ hydrolysis and therefore Ca^{2+} release is possible when some vicinal thiols are cross-linked. Here we report that the thiol oxidant peroxovanadate inhibits the specific Ca^{2+} release pathway. In mitochondria, peroxovanadate causes a complete loss of reduced glutathione, which is not accompanied by formation of glutathione disulfide, and a partial loss of protein thiols. In model reactions, peroxovanadate oxidizes reduced glutathione predominantly to the sulfonate derivative, but does not react with glutathione disulfide. When the vicinal thiols relevant for Ca^{2+} release are cross-linked, Ca^{2+} release is no longer inhibited by peroxovanadate. Conversely, pretreatment of mitochondria with peroxovanadate makes them insensitive to compounds promoting the disulfide state. These results suggest that peroxovanadate inhibits the prooxidant-induced Ca^{2+} release from mitochondria by (i) depleting mitochondria of reduced glutathione and (ii) oxidizing the vicinal thiols relevant for Ca^{2+} release to a state higher than disulfide, presumably the sulfonate state. The findings provide further insight into the regulation of Ca^{2+} release from intact mitochondria, and may be relevant for a better understanding of the action of peroxovanadate in cells, where the compound can be insulin mimetic.

Key words. Vicinal thiol; intactness; glutathione; peroxynitrite; gliotoxin; disulfide; glutathione oxidation; sulfonate.

Cytosolic Ca^{2+} controls key cellular events, such as muscle contraction, secretion, neuronal activity, and modulation of hormone action. Traditionally, mitochondria were regarded mainly as a safety device against potentially toxic increases of cytosolic Ca^{2+} [reviewed in ref. 1] because their affinity for Ca^{2+} was thought to render them insufficient to compete with other Ca^{2+} transport systems located in the nucleus, the endoplasmic reticulum, and the plasma membrane. However, there is now compelling evidence that the mitochondrial contribution to Ca^{2+} buffering is physiologically relevant. It plays an important role in shaping physiological Ca^{2+} transients and maintaining cellular Ca^{2+} homeostasis [2–4], and provides an efficient mechanism for modulating the activity of mitochondrial enzymes upon cell stimulation [5, 6].

Mitochondria take up and release Ca^{2+} via different pathways. As a consequence, Ca^{2+} can be 'cycled' across their inner membrane [7]. The mitochondrial membrane potential ($\Delta\Psi$), which is maintained either by respiration or by ATP hydrolysis, drives Ca^{2+} into mitochondria. Principally, Ca^{2+} can leave mitochondria in four ways: (i) by nonspecific leakage through the inner membrane, (ii) by reversal of the influx carrier due to a decreased $\Delta\Psi$, and (iii) via Na^+-dependent or (iv) Na^+-independent release

* Corresponding author.
‡ Present address: Institute of Veterinary Virology, University of Bern, 3012 Berne, Switzerland.

pathways. Probably only the latter two are physiologically relevant, because they operate when $D\Psi$ is high. The Na^+-dependent pathway predominates in mitochondria of heart, brain, and other excitable tissues. The Na^+-independent pathway is important in organs such as liver, kidney, or lung, probably exchanges Ca^{2+} with H^+, and is linked to the redox state of mitochondrial pyridine nucleotides [8, and references therein]. This pathway is stimulated by the prooxidants H_2O_2 or *t*-butylhydroperoxide [9, 10], and requires an intramitochondrial Ca^{2+}-dependent hydrolysis of NAD^+ [reviewed in ref. 11]. Hydrolysis is only possible when some vicinal thiols are cross-linked, either by oxidation [12] or by reaction with phenylarsine oxide [13], and is prevented when these thiols are alkylated [14].

Oxidized vanadate compounds cause numerous modifications or responses in cells, such as protein tyrosine phosphatase inhibition, tyrosine kinase activation, increased glucose transport, elevation of intracellular Ca^{2+}, or phospholipase activation [for review, see ref. 15]. Best known are the insulin-mimetic properties of peroxovanadates [16]. Since the increase in cytosolic Ca^{2+} induced by vanadate involves the oxidation of thiols [17] and mitochondrial Ca^{2+} release is controlled by vicinal thiols, we analyzed the possible modification of mitochondrial Ca^{2+} homeostasis by peroxovanadate. We find that peroxovanadate prevents the prooxidant-induced Ca^{2+} release by oxidizing reduced glutathione (GSH). Peroxovanadate most likely also oxidizes other thiols to sulfonates because it partially depletes mitochondria of protein thiols and inhibits the Ca^{2+} release triggered by thiol oxidation and cross-linking.

Materials and methods

Materials

α-Cyano-4-hydroxy-cinnamic acid was obtained from Fluka (Buchs, Switzerland). Peroxynitrite was provided by Dr. R. Kissner [18], and stored at −80 °C. Its concentration was determined photometrically at 302 nm in 0.1 M sodium hydroxide ($\varepsilon_{mM} = 1.67$) [19] daily prior to use. Peroxovanadate (40 mM) was synthesized by adding 1 ml of 100 mM vanadate to 1.5 ml of 90 mM H_2O_2. After 15 min, the pH was adjusted to 7.0. Excess H_2O_2 was removed with catalase (1000 units/ml). The combination of vanadate and H_2O_2 under physiological conditions generates several different peroxovanadium species in equilibrium with one another depending upon the pH of the solution, and the concentration of both vanadate and H_2O_2 [20]. At the concentrations of vanadate and hydrogen peroxide used here, monoperoxo-, diperoxo-, and to a smaller extent triperoxo-vanadium forms are synthesized. 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 [21]. The protein content was determined by the Biuret method with bovine serum albumin as standard.

Standard incubation procedure

Mitochondria (1 mg of protein/ml) were incubated at 25 °C with continuous stirring and oxygenation in 3 ml of 210 mM mannitol, 70 mM sucrose, 40 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), pH 7.2. The high concentration of Hepes was used to prevent pH changes upon addition of the alkaline peroxynitrite solution.

Determination of Ca^{2+} uptake and release by mitochondria

The standard incubation procedure was followed. After addition of rotenone (5 μM) and K^+-succinate (2.5 mM), mitochondria were loaded with Ca^{2+} (40 nmol/mg of mitochondrial protein). Its movement across the inner mitochondrial membrane was monitored by a spectrophotometric technique in the presence of 50 μM arsenazo III in an Aminco DW2A spectrophotometer at 685−675 nm [22].

Determination of mitochondrial swelling

Mitochondria were incubated according to the standard procedure. After addition of rotenone (5 μM) and K^+-succinate (2.5 mM) they were loaded with Ca^{2+}. Swelling was followed in an Aminco DW2A spectrophotometer at 540 nm.

Determination of mitochondrial glutathione

GSH and glutathione disulfide (GSSG) were determined by high-performance liquid chromatography according to Fariss et al. [23] and Reed et al. [24].

Protein sulfhydryl oxidation assay

The amount of mitochondrial membrane thiol was measured using 5,5′-dithiobis (2-nitrobenzoic acid) at 412 nm in a Kontron Uvikon model 922 spectrophotometer, as described previously [25].

Matrix-assisted laser disorption/ionization (MALDI) mass spectrometry

Experiments were performed on a home-built 2 m linear time-of-flight mass spectrometer equipped with a nitrogen laser [26]. Samples were prepared using the two-layer technique [27] and spectra were recorded in positive-ion mode using α-cyano-4-hydroxy-cinnamic acid as matrix. Relative signal intensities were referenced to internal standard, and signal integrals were obtained using commercial software.

Measurement of H_2O_2 production

The production of H_2O_2 in mitochondria was measured fluorometrically by the scopoletin method [28] in an Aminco-Bowman 4-8202 spectrofluorometer with excitation at 350 nm and emission at 460 nm.

Results

Peroxovanadate inhibits Ca^{2+} release from and swelling of mitochondria

Mitochondria take up and release Ca^{2+} via different routes, which operate simultaneously and can be manipulated individually. Uptake can be blocked by, e.g., ruthenium red, and release from intact mitochondria can be stimulated by prooxidants, e.g., *t*-butylhydroperoxides [9]. Figure 1 illustrates this with succinate-energized mitochondria. When net Ca^{2+} uptake is completed and a steady-state distribution of Ca^{2+} is achieved, addition of ruthenium red causes Ca^{2+} release (fig. 1, trace a). Release is stimulated by t-butylhydroperoxide (fig. 1, trace b), but in the presence of peroxovanadate this stimulation does not take place (fig. 1, trace c). Mitochondria treated with peroxovanadate, washed, and then tested for their ability to release Ca^{2+} upon t-butylhydroperoxide addition were still completely unresponsive to this compound (not shown). When Ca^{2+} release was triggered by phenylarsine oxide, a molecule that cross-links vicinal thiols, addition of peroxovanadate does not result in inhibition (fig. 2). Note that when phenylarsine oxide is added after peroxovanadate, Ca^{2+} release is inhibited (see fig. 8a).

When intact mitochondria are stimulated to release Ca^{2+} and take it up again ('cycle' Ca^{2+}), they swell and eventually become irreversibly damaged [21]. Swelling induced by Ca^{2+} in combination with *t*-butylhydroperoxide is dose-dependently prevented by peroxovanadate (fig. 3).

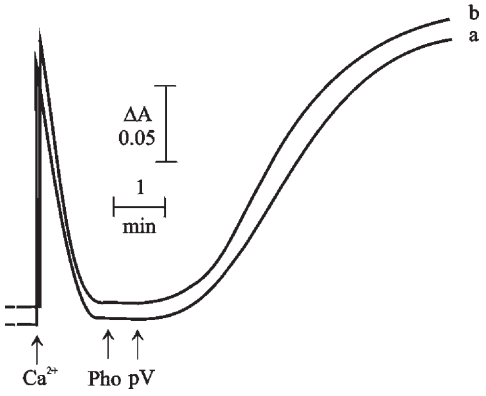

Figure 2. Ca^{2+} release from mitochondria induced by phenylarsine oxide. Mitochondria were incubated under standard conditions in the presence of the Ca^{2+} indicator arsenazo III and loaded with Ca^{2+} (added at the first arrow) as in figure 1. At the second arrow, the vicinal thiol cross-linker phenylarsine oxide (40 μM) was added (trace a), followed (third arrow) by 1 mM peroxovanadate (trace b).

Mechanism of peroxovanadate inhibition

t-Butylhydroperoxide stimulates Ca^{2+} release when glutathione peroxidase, glutathione reductase, and the energy-linked transhydrogenase reduce the peroxide with NADH [9]. This enzyme cascade engages glutathione. Since peroxovanadate inhibited the peroxide-stimulated Ca^{2+} release and subsequent swelling, we set out to understand the mechanism of peroxovanadate action.

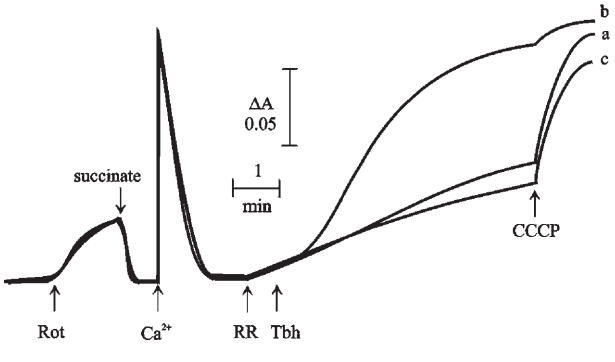

Figure 1. Peroxovanadate-inhibited Ca^{2+} release from mitochondria. Mitochondria were incubated under standard conditions in the presence of the Ca^{2+} indicator arsenazo III. The movement of Ca^{2+} across the inner mitochondrial membrane was followed in an Aminco DW2A spectrophotometer at 685–675 nm. At the arrows, the following additions were made: 5 μM rotenone (Rot); 2.5 mM K^+-succinate (succinate); 40 nmol Ca^{2+} (Ca^{2+}); ruthenium red (2 nmol/mg of protein) (RR); 100 μM *t*-butylhydroperoxide (Tbh); 1 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). Trace a, without *t*-butylhydroperoxide; trace b, with *t*-butylhydroperoxide; trace c, as trace b but in the presence of 1 mM peroxovanadate.

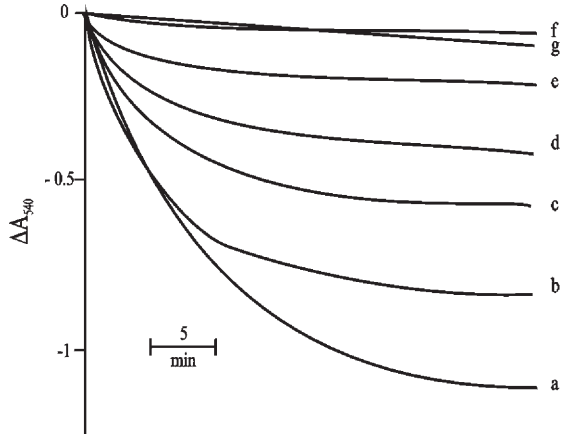

Figure 3. Peroxovanadate-induced swelling of mitochondria. Mitochondria were incubated under standard conditions in the presence of 5 μM rotenone, 2.5 K^+-succinate, and Ca^{2+} (40 nmol/mg of mitochondrial protein). To initiate swelling, 100 μM *t*-butylhydroperoxide was added. Swelling was followed in an Aminco DW2A spectrophotometer at 540 nm. Traces a–f, in the presence of 0, 50, 100, 200, 500, 1000 μM peroxovanadate, respectively; trace g, in the absence of *t*-butylhydroperoxide and peroxovanadate.

Peroxovanadate oxidizes glutathione to the sulfonate state

In mitochondria exposed for 1 min to 1 mM peroxovanadate no GSH (0 nmol GSH vs 2.75 nmole GSH in control mitochondria), and no GSSG (0 nmol GSSG vs 0.19 nmol GSSG in control mitochondria) is detectable. The activities of mitochondrial glutathione peroxidase or of purchased glutathione reductase, both measured according to Cardoso et al. [29], were not affected by peroxovanadate (results not shown).

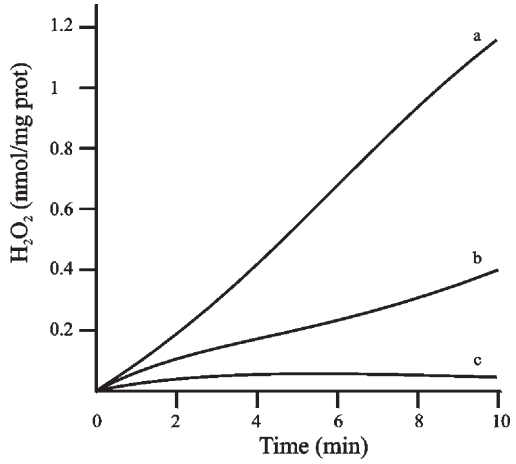

Figure 4. Formation of hydrogen peroxide in mitochondria. Mitochondria (0.5 mg/ml) were incubated under standard conditions in the presence of rotenone and succinate, as in figure 1. H_2O_2 was measured as described in Materials and methods. Trace a, mitochondria were treated for 1 min with 1 mM peroxovanadate, then washed and resuspended; trace b, untreated mitochondria (control); trace c, untreated mitochondria incubated in the absence of succinate.

As mentioned above, mitochondria reduce peroxides in a glutathione-requiring enzyme cascade. In succinate-energized mitochondria exposed to peroxovanadate, much more H_2O_2 is detected than in control mitochondria (fig. 4), supporting the notion that peroxovanadate causes a loss of mitochondrial GSH. In the absence of succinate, mitochondria did not produce H_2O_2 (fig. 4).

Since in mitochondria exposed to peroxovanadate the loss of GSH was not accompanied by formation of GSSG, we probed the fate of glutathione by MALDI mass spectrometry. When we mixed 40 mM peroxovanadate and 8 mM GSH in a test tube and analyzed 10 min later, we found predominantly glutathione oxidized to the sulfonate (GSO_3H) and a small amount of GSSG (fig. 5). Mass signal intensities of GSH, GSO_3H, and GSSG were integrated and referenced to internal standard (matrix, arbitrarily set to 100% for normalization). The diagram shows that the sulfonate form GSO_3H contributes extensively to the sample after oxidation (about one order of magnitude), whereas signals deciphering the disulfide form GSSG remain largely unchanged (see figure captions for calculated values). In a separate enzymatic assay we found that peroxovanadate does not oxidize GSSG (fig. 6). These results suggest that peroxovanadate converts GSH in a significant reaction directly to GSO_3H, and in a side reaction to GSSG.

Peroxovanadate oxidizes mitochondrial protein thiols

In mitochondria exposed to peroxovanadate, the content of membrane thiols decreases dose-dependently (fig. 7). We have previously shown that vicinal thiols control (i) the hydrolysis of mitochondrial NAD^+ and thereby (ii) the release of Ca^{2+}: when the vicinal thiols are in the dithiol

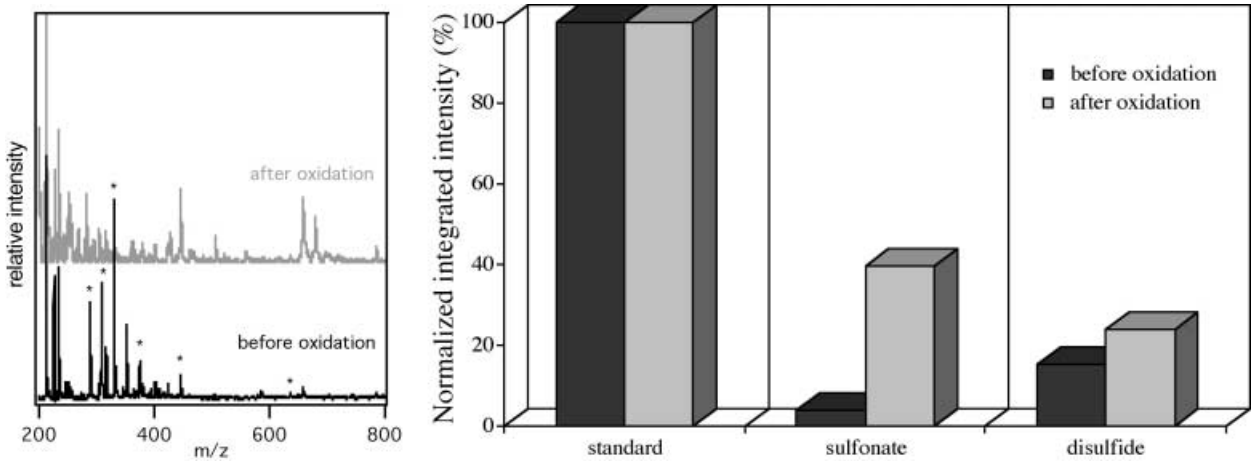

Figure 5. MALDI mass analysis of the reaction between reduced glutathione (GSH) and peroxovanadate. Left panel: positive-ion mode MALDI mass spectra of GSH before (lower trace) and after (upper trace) oxidation with peroxovanadate. Analytes (asterix) have been identified by their protonated ($A+H^+$) or sodiated ($A+Na^+$) species. Right panel: integrated signal intensities, as referenced to internal standard (matrix signals, normalized to 100%). Relative contributions are assigned for the following species: 3.8% sulfonate/15.2% disulfide before oxidation (black bars); 39.5% sulfonate/23.7% disulfide after oxidation (gray bars). Qualitatively, the amount of disulfide in the sample stays the same, whereas the amount of sulfonate increases by more than one order of magnitude.

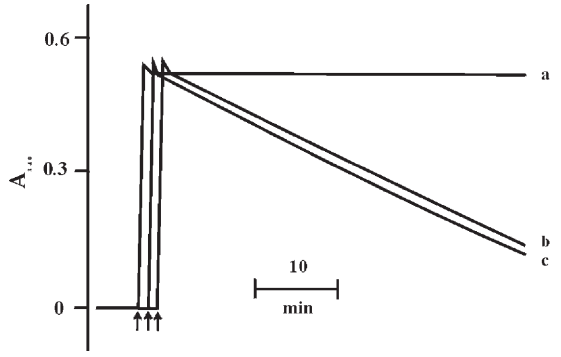

Figure 6. Peroxovanadate does not oxidize the disulfide form of glutathione (GSSG). The possible oxidation of GSSG was tested in an assay using the glutathione reductase-mediated oxidation of NADPH by GSSG. Glutathione reductase (1 unit/ml) was suspended in 50 mM phosphate buffer, pH 7, containing 1 mM EDTA, with or without peroxovanadate. At the arrow, 100 µM NADPH was added. Its oxidation was followed spectrophotometrically at 340 nm. Trace a, no GSSG present; trace b, 1 mM GSSG present; trace c, as trace b but 1 mM peroxovanadate also present.

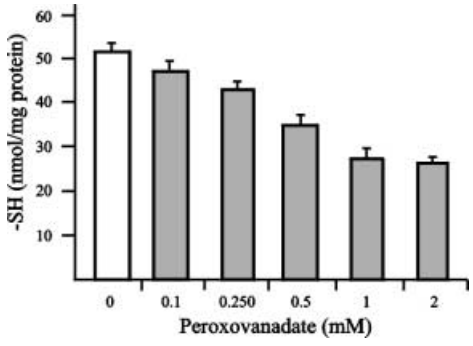

Figure 7. Peroxovanadate oxidizes mitochondrial protein thiols. Mitochondria were incubated for 5 min under standard conditions in the presence of 500 µM EGTA with or without peroxovanadate at the indicated concentrations. Protein thiols were measured (average ± SD of three experiments) as described in Materials and methods. The control protein thiol content is 52 ± 3.1 (nmol/mg protein).

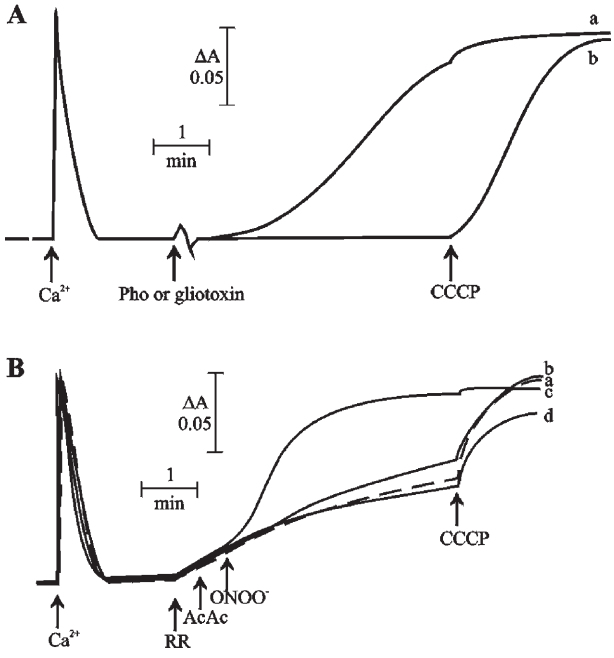

Figure 8. Peroxovanadate inhibits Ca^{2+} release from mitochondria. Mitochondria were incubated under standard conditions in the presence of the Ca^{2+} indicator arsenazo III and with the subsequent addition of 5 µM rotenone and 2.5 mM K^+-succinate. Ca^{2+} release was triggered by the thiol oxidants/cross-linkers gliotoxin, phenylarsine oxide, or peroxynitrite. The movement of Ca^{2+} across the inner mitochondrial membrane was followed as in figure 1. (A) Ca^{2+} release triggered by gliotoxin or phenylarsine oxide. At the arrows, the following additions were made: 40 nmol Ca^{2+} (Ca^{2+}); 8 µM phenylarsine oxide (Pho) or 50 µM gliotoxin; 1 µM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). Trace a, without peroxovanadate; trace b, with 1 mM peroxovanadate. (B) Ca^{2+} release triggered by peroxynitrite. At the arrows, the following additions were made: 40 nmol Ca^{2+}/mg of mitochondrial protein (Ca^{2+}), ruthenium red (2 nmol/mg of mitochondrial protein) (RR); 1 mM acetoacetate (AcAc); 500 µM peroxynitrite ($ONOO^-$); 1 µM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). Traces a – c, without peroxovanadate; trace d, with 1 mM peroxovanadate. Traces b – d, with acetoacetate, and traces c and d with peroxynitrite.

state or are alkylated they inhibit, and when they are cross-linked and/or in the disulfide state they promote the two events. Cross-linking can be achieved with phenylarsine oxide [13], and oxidation to the disulfide state with gliotoxin [12] or peroxynitrite [30]. We found that peroxovanadate inhibits Ca^{2+} release induced by phenylarsine oxide, gliotoxin, or peroxynitrite (fig. 8). The peroxovanadate-induced thiol modification is stable: as with *t*-butylhydroperoxide, treatment of mitochondria with peroxovanadate followed by washing this compound out left mitochondria insensitive to phenylarsine oxide (result not shown).

Peroxovanadate versus other transition metal oxides

Peroxocompounds of some other transition metals have biological activities similar to those of peroxovanadate. We therefore also tested peroxotungsten and peroxomolybdenum. These compounds had a similar but less potent influence on Ca^{2+} release compared to peroxovanadate. Vanadate was about half as potent as peroxovanadate, which may relate to H_2O_2 formation in mitochondria [31] followed by in situ formation of peroxovanadate.

Discussion

The prooxidant-stimulated release of Ca^{2+} from intact mitochondria is a tightly controlled process. It takes place when intramitochondrial pyridine nucleotides are oxidized, and when NAD^+ is hydrolyzed to ADPribose and nicotinamide [10]. The reduction of a hydroperoxide in mitochondria and therefore the provision of NAD^+ requires glutathione [32], and hydrolysis of NAD^+ requires

the cross-linking and/or oxidation of critical vicinal thiols [12, 13, 30]. We report here that peroxovanadate inhibits the prooxidant-stimulated release of Ca^{2+} from mitochondria.

Since mitochondria produce H_2O_2 [31], our findings with peroxovanadate are consistent with the report [33] that vanadate causes mitochondrial Ca^{2+} overload in hepatocytes.

Peroxovanadate blocks the prooxidant-stimulated Ca^{2+} release pathway in mitochondria at two sites (fig. 9). One is GSH/GSSG, which are no longer present in detectable amounts when mitochondria are exposed to peroxovanadate. The finding that GSH and GSSG disappear from mitochondria, and that peroxovanadate can convert GSH largely to GSO_3H makes it highly likely that in mitochondria also GSO_3H is formed in the presence of peroxovanadate. The other site of blockade comprises the vicinal thiols which control NAD^+ hydrolysis. In mitochondria exposed to peroxovanadate, the number of membrane thiols decreases. We previously reported that thiol alkylation by 4-hydroxynonenal causes a persistent inhibition of the Ca^{2+} release pathway [14]. Others have reported that peroxovanadate converts cysteine residues to cysteic acid ($Cys[SO_3H]$) residues in proteins [34]. We presume that the inactivation of the Ca^{2+} release pathway in the experiments that employed peroxovanadate-pretreated mitochondria and phenylarsine oxide, gliotoxin, or peroxynitrite (fig. 8) is caused by persistent oxidation of the vicinal thiols to cysteic acid residues by peroxovanadate.

When peroxovanadate is added to mitochondria whose Ca^{2+} release-relevant vicinal thiols are already cross-linked, release is not blocked. This suggests that, as in the case of GSSG, the cross-linked thiols are not further oxidized by peroxovanadate.

Ca^{2+} release from mitochondria can be induced by a variety of prooxidants. These compounds initially leave the mitochondria intact. However, excessive Ca^{2+} cycling (continuous release and uptake of Ca^{2+} by mitochondria) may lead to their damage, e.g., decrease in $\Delta\Psi$, fast Ca^{2+}

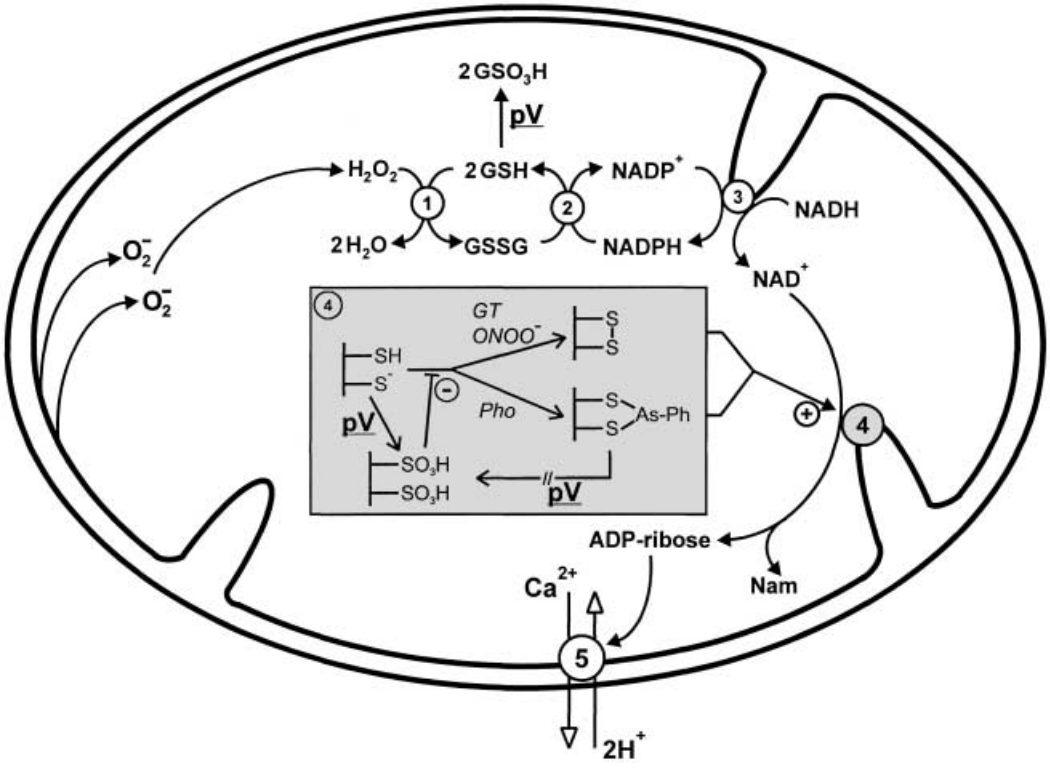

Figure 9. How peroxovanadate inhibits Ca^{2+} release from mitochondria. Mitochondria contain a specific Ca^{2+} release pathway that operates with preservation of the membrane potential. This pathway is stimulated by the prooxidants H_2O_2 or *t*-butylhydroperoxide, and requires an intramitochondrial, Ca^{2+}-dependent hydrolysis of NAD^+. Hydrolysis is only possible when some vicinal thiols are cross-linked, either by oxidation or by reaction with phenylarsine oxide, and is prevented when these thiols are alkylated. Peroxovanadate (pV) inhibits the prooxidant-induced Ca^{2+} release by depleting mitochondria of reduced glutathione (GSH), which it oxidizes to the sulfonate (GSO_3H). Peroxovanadate also inhibits Ca^{2+} release initiated by cross-linking/oxidation of vicinal thiols with gliotoxin (GT), peroxynitrite ($ONOO^-$), or phenylarsine oxide (Pho), presumably because it oxidizes the vicinal thiols to the sulfonate ($-SO_3H$) state. Peroxovanadate does not oxidize the disulfide form of glutathione (not shown), nor the cross-linked vicinal thiols to the sulfonates. ①, ②, and ③ are the enzyme cascade that links a hydroperoxide to NADH and which consists of ① glutathione peroxidase, ② glutathione reductase, and ③ the energy-linked transhydrogenase; ④ is the NAD^+ hydrolyzing system; ⑤ is the (unidentified) Ca^{2+} release channel. Nam, nicotinamide, ⊕, stimulated; ⊖, inhibited.

release, and impairment of ATP synthesis. However, when Ca^{2+} cycling is prevented by Ca^{2+} chelators or by inhibition of Ca^{2+} uptake with ruthenium red, prooxidants still induce Ca^{2+} release but mitochondria stay intact [8, 21]. Formation of a nonspecific permeability transition pore in the inner mitochondrial membrane has been suggested to participate in the Ca^{2+} release mechanisms [35, and references therein]. However, the specific, prooxidant-induced Ca^{2+} release does not require the formation of a pore provided Ca^{2+} cycling is inhibited [21].

Precise modulation of the mitochondrial pyridine nucleotide redox state is important for mitochondrial as well as cytosolic Ca^{2+} regulation, and for cellular energy demand. Thus, oscillation of cytosolic Ca^{2+} induced by IP_3-dependent hormones is effectively transmitted into oscillations of mitochondrial Ca^{2+} concentration paralleled by changes in the mitochondrial pyridine nucleotide redox state [36]. In rat pancreatic islets, the nutrient D-glucose triggers the fall in the pyridine nucleotide redox state, thus decreasing the mitochondrial $NADH/NAD^+$ ratio, which favors the exit of Ca^{2+} from mitochondria [37]. However, the exact mechanism of the specific Ca^{2+} release pathway from mitochondria and its role in the modulation of cytosolic and mitochondrial Ca^{2+} homeostasis in response to various stimuli still needs to be clarified.

Peroxovanadium compounds but not vanadate itself [38] are known for their insulin-mimetic properties [for review, see ref. 15]. Whether the previously reported biological actions of vanadate (see Introduction) are due to intracellularly formed peroxovanadate [39], and whether insulin mimesis of peroxovanadates relates to mitochondrial Ca^{2+} homeostasis remains to be established.

Acknowledgement. The generous gift of peroxynitrite provided by Dr. R. Kissner is gratefully acknowledged.

1 Richter C. and Kass G. E. N. (1991) Oxidative stress in mitochondria – its relationship to cellular Ca^{2+} homeostasis, cell death, proliferation, and differentiation. Chem. Biol. Interact. **77:** 1–23
2 Rizzuto R., Bastianutto C., Brini M., Murgia M. and Pozzan T. (1994) Mitochondrial Ca^{2+} homeostasis in intact cells. J. Cell Biol. **126:** 1183–1194
3 Jouaville L. S., Ichas F., Holmuhamedov E. L., Camacho P. and Lechleiter J. D. (1995) Synchronization of calcium waves by mitochondrial substrates in *Xenopus laevis* oocytes. Nature **377:** 438–441
4 Duchen M. R. (2000) Mitochondria and Ca^{2+} in cell physiology and pathophysiology. Cell Calcium **28:** 339–348
5 McCormack J. G., Halestrap A. P. and Denton R. M. (1990) Role of calcium ions in regulation of mammalian intramitochondrial metabolism. Physiol. Rev. **70:** 391–425
6 Richter C. (1992) Mitochondrial calcium transport. In: Molecular Mechanisms in Bioenergetics, vol. 23, pp. 349–358, Ernster L. (ed.), Elsevier, Amsterdam
7 Carafoli E. (1979) The calcium cycle of mitochondria. FEBS Lett. **104:** 1–5
8 Richter C., Schlegel J. and Schweizer M. (1992) Prooxidant-induced Ca^{2+} release from liver mitochondria – specific versus nonspecific pathways. Ann. NY Acad. Sci. **663:** 262–268
9 Lötscher H. R., Winterhalter K. H., Carafoli E. and Richter C. (1979) Hydroperoxides can modulate the redox state of pyridine nucleotides and the calcium balance in rat liver mitochondria. Proc. Natl. Acad. Sci. USA **76:** 4340–4344
10 Lötscher H. R., Winterhalter K. H., Carafoli E. and Richter C. (1980) Hydroperoxide-induced loss of pyridine nucleotides and release of calcium from rat liver mitochondria. J. Biol. Chem. **255:** 9325–9330
11 Richter C. and Schlegel J. (1993) Mitochondrial calcium release induced by prooxidants. Toxicol. Lett. **67:** 119–127
12 Schweizer M. and Richter C. (1994) Gliotoxin stimulates Ca^{2+} release from intact rat liver mitochondria. Biochemistry **33:** 13401–13405
13 Schweizer M., Durrer P. and Richter C. (1994) Phenylarsine oxide stimulates pyridine nucleotide-linked Ca^{2+} release from rat liver mitochondria. Biochem. Pharmacol. **48:** 967–973
14 Klein S. D., Schweizer M. and Richter C. (1996) Inhibition of the pyridine nucleotide-linked mitochondrial Ca^{2+} release by 4-hydroxynonenal: the role of thiolate-disulfide conversion. Redox Rep. **2:** 353–358
15 Bevan A. P., Drake P. G., Yale J. F., Shaver A. and Posner B. I. (1995) Peroxovanadium compounds: biological actions and mechanism of insulin-mimesis. Mol. Cell. Biochem. **153:** 49–58
16 Posner B. I., Faure R., Burgess J. W., Bevan A. P., Lachance D., Zhang-Sun G. et al. (1994) Peroxovanadium compounds: a new class of potent phosphotyrosine phosphatase inhibitors which are insulin mimetics. J. Biol. Chem. **269:** 4596–4604
17 Ehring G. R., Kerschbaum H. H., Fanger C. M., Eder C., Rauer H. and Cahalan M. D. (2000) Vanadate induces calcium signaling, Ca^{2+} release-activated Ca^{2+} channel activation, and gene expression in T lymphocytes and RBL-2H3 mast cells via thiol oxidation. J. Immunol. **164:** 679–687
18 Koppenol W. H., Kissner R. and Beckman J. S. (1996) Syntheses of peroxynitrite: To go with the flow or on solid grounds? Methods Enzymol. **269**: 296-302
19 Beckman J. S., Chen J., Ischiropoulos H. and Crow J. P. (1994) Oxidative Chemistry of Peroxynitrite. Methods Enzymol. **233:** 229–240
20 Shaver A., Ng J. B., Hall D. A. and Posner B. I. (1995) The chemistry of peroxovanadium compounds relevant to insulin mimesis. Mol. Cell. Biochem. **153:** 5–15
21 Schlegel J., Schweizer M. and Richter C. (1992) Pore formation is not required for the hydroperoxide-induced Ca^{2+} release from rat liver mitochondria. Biochem. J. **285:** 65–69
22 Frei B., Winterhalter K. H. and Richter C. (1985) Mechanism of alloxan-induced calcium release from liver mitochondria. J. Biol. Chem. **260:** 7394–7401
23 Fariss M. W., Olafsdottir K. and Reed D. J. (1984) Extracellular calcium protects isolated rat hepatocytes from injury. Biochem. Biophys. Res. Commun. **121:** 102–110
24 Reed D. J., Babson J. R., Beatty P. W., Brodie A. E., Ellis W. W. and Potter D. W. (1980) High-performance liquid chromatography analysis of nanomole levels of glutathione, glutathione disulfide, and related thiols and disulfides. Anal. Biochem. **106:** 55–62
25 Kowaltowski A. J., Vercesi A. E. and Castilho R. F. (1997) Mitochondrial membrane protein thiol reactivity with N-ethylmaleimide or mersalyl is modified by Ca^{2+}: correlation with mitochondrial permeability transition. Biochim. Biophys. Acta **1318:** 395–402
26 Friess S. D. and Zenobi R. (2001) Protein structure information from mass spectrometry? Selective titration of arginine residues by sulfonates. J. Am. Soc. Mass Spectrom. **12:** 810–818
27 Görisch H. (1988) Drop dialysis: time course of salt and protein exchange. Anal. Biochem. **173:** 393–398
28 Loschen G., Azzi A. and Flohé L. (1973) Mitochondrial H_2O_2 formation: relationship with energy conservation. FEBS Lett. **33:** 84–87

29 Cardoso S. M., Pereira C. and Oliveira C. R. (1998) The protective effect of vitamin E, idebenone and reduced glutathione on free radical mediated injury in rat brain synaptosomes. Biochem. Biophys. Res. Commun. **246:** 703–710
30 Schweizer M. and Richter C. (1996) Peroxynitrite stimulates the pyridine nucleotide-linked Ca^{2+} release from intact rat liver mitochondria. Biochemistry **35:** 4524–4528
31 Chance B., Sies H. and Boveris A. (1979) Hydroperoxide metabolism in mammalian organs. Physiol. Rev. **59:** 527–605
32 Frei B., Winterhalter K. H. and Richter C. (1985) Quantitative and mechanistic aspects of the hydroperoxide-induced release of Ca^{2+} from rat liver mitochondria. Eur. J. Biochem. **149:** 633–639
33 Richelmi P., Mirabelli F., Salis A., Finardi G., Berte F. and Bellomo G. (1989) On the role of mitochondria in cell injury caused by vanadate-induced Ca^{2+} overload. Toxicology **57:** 29–44
34 Huyer G., Liu S., Kelly J., Moffat J., Payette P., Kennedy B. et al. (1997) Mechanism of inhibition of protein-tyrosine phosphatases by vanadate and pervanadate. J. Biol. Chem. **272:** 843–851
35 Zoratti M. and Szabo, I. (1995) The mitochondrial permeability transition. Biochim. Biophys. Acta **1241:** 139–176
36 Hajnóczky G., Robbgaspers L.D., Seitz M.B. and Thomas, A.P. (1995) Decoding of cytosolic calcium oscillations in the mitochondria. Cell **82:** 415–424
37 Ramirez R., Sener A. and Malaisse W. J. (1995) Hexose metabolism in pancreatic islets: regulation of the mitochondrial NADH/NAD^+ ratio. Biochem. Mol. Med. **55:** 1–7
38 Lonnroth P., Eriksson J. W., Posner B. I. and Smith U. (1993) Peroxovanadate but not vanadate exerts insulin-like effects in human adipocytes. Diabetologia **36:** 113–116
39 Cuncic C., Detich N., Ethier D., Tracey A. S., Gresser M. J. and Ramachandran C. (1999) Vanadate inhibition of protein tyrosine phosphatases in Jurkat cells: modulation by redox state. J. Biol. Inorg. Chem. **4:** 354–359