THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 269, No. 20, Issue of May 20, pp. 14546-14552, 1994
Printed in U.S.A.

Role of Mitochondrial Ca^{2+} in the Oxidative Stress-induced Dissipation of the Mitochondrial Membrane Potential

STUDIES IN ISOLATED PROXIMAL TUBULAR CELLS USING THE NEPHROTOXIN 1,2-DICHLOROVINYL-L-CYSTEINE*

(Received for publication, October 1, 1993, and in revised form, February 7, 1994)

Bob van de Water‡, J. Paul Zoeteweij, Hans J. G. M. de Bont, Gerard J. Mulder, and J. Fred Nagelkerke

From the Division of Toxicology, Leiden Amsterdam Center for Drug Research, Leiden University, 2300 RA Leiden, The Netherlands

The relationship between mitochondrial Ca^{2+}, oxidative stress, and a dissipation of the mitochondrial membrane potential ($\Delta\psi$) was investigated in proximal tubular kidney cells. Freshly isolated proximal tubular cells from rat kidney were exposed to the nephrotoxin 1,2-dichlorovinyl-L-cysteine (DCVC). DCVC stimulated the formation of hydroperoxides as determined by flow cytometry using the hydroperoxide-sensitive compound dichlorofluorescin. This was prevented by the antioxidant diphenylphenylenediamine (DPPD) and the iron chelator desferrioxamine. Studies in individual cells with video-intensified fluorescence microscopy showed that a DCVC-induced increase in the intracellular free calcium concentration ($[Ca^{2+}]_i$) was accompanied by an increase in the mitochondrial free calcium concentration ($[Ca^{2+}]_m$). The latter increase was selectively prevented by an inhibitor of the mitochondrial calcium uniporter, ruthenium red (RR). Chelation of cellular Ca^{2+} with EGTA acetoxymethyl ester (EGTA/AM) completely prevented the formation of hydroperoxides, whereas inhibition of the uptake of Ca^{2+} by the mitochondria with RR reduced it. This indicates that the increase in $[Ca^{2+}]_m$ is important for the induction of oxidative stress by DCVC. DPPD and desferrioxamine did not protect against a DCVC-induced increase in $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$, indicating that oxidative stress is the consequence rather than the cause of the cellular calcium perturbations. DCVC decreased $\Delta\psi$ and caused cell death; both effects were clearly delayed by EGTA/AM and RR, although they could not prevent a decrease in $\Delta\psi$. The latter decrease was completely prevented by inhibition of the β-lyase-mediated metabolism of DCVC with aminooxyacetic acid. Like EGTA/AM, inhibition of oxidative stress with DPPD and desferrioxamine delayed the decrease in $\Delta\psi$. This strongly suggests that the decrease in $\Delta\psi$ caused by metabolites of DCVC directly is potentiated by Ca^{2+}-dependent DCVC-induced hydroperoxide formation. The importance of both hydroperoxide formation and mitochondrial damage in DCVC-induced cell killing is discussed.

Exposure of isolated mitochondria to a high concentration of calcium leads to mitochondrial injury (1–4). Calcium-induced mitochondrial injury can be prevented by antioxidants (5–8), suggesting that oxidative stress may be an important event in its development. Indeed, exposure of heart mitochondria to high calcium levels was reported to result in the formation of reactive oxygen species (ROS)[1] (9, 10). Recently, it was shown in isolated mitochondria that calcium-induced oxidative stress can be prevented by an inhibitor of the mitochondrial Ca^{2+} uniporter, ruthenium red, suggesting that calcium uptake in the mitochondrial matrix is required for ROS formation (10).

Exogenous ROS cause severe injury in isolated mitochondria (11–13). Such injury was also observed in isolated liver mitochondria after endogenous ROS formation (6). This raises the question whether such an effect may play a role in the cytotoxicity of xenobiotics in intact cells. However, evidence for a role of calcium-dependent ROS formation in mitochondria in intact cells has not been documented yet. We decided to study this with 1,2-dichlorovinyl-L-cysteine (DCVC) in isolated proximal tubular cells from rat.

DCVC belongs to the group of nephrotoxic haloalkenyl-L-cysteine *S*-conjugates and is often used as a model compound to investigate the mechanism of toxicity of these conjugates (14–16). They are activated by renal cysteine-*S*-conjugate β-lyase to thiol-containing electrophilic reactive metabolites (17, 18), which bind covalently to macromolecules, thereby initiating cellular damage (19). Inhibition of cysteine-*S*-conjugate β-lyase prevents covalent binding and thereby irreversible cellular injury (19). Several intracellular events are initiated after metabolism of DCVC. Stevens and co-workers (19) showed that oxidative stress is involved in the toxicity of DCVC, which was later confirmed by us (22) and others (21). In addition, we showed that DCVC increases the intracellular free calcium concentration ($[Ca^{2+}]_i$) and decreases the mitochondrial membrane potential ($\Delta\psi$) in freshly isolated rat renal proximal tubular cells (20). Chelation of intracellular Ca^{2+} clearly protects against an initial decrease in $\Delta\psi$ as well as cell death (20). These results, in combination with those obtained with isolated mitochondria described above, prompted us to investigate the relationship between oxidative stress, perturbation of cytosolic and mitochondrial calcium, and mitochondrial injury in DCVC-induced cytotoxicity. Our findings indicate that exposure of PTC to DCVC results in increased influx of Ca^{2+} into the mitochondrial matrix, which induces the formation of hydroperox-

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Div. of Toxicology, Sylvius Laboratories, P. O. Box 9503, 2300 RA Leiden, The Netherlands. Tel.: 31-71-276039; Fax: 31-71-276292.

[1] The abbreviations used are: ROS, reactive oxygen species; DCVC, 1,2-dichlorovinyl-L-cysteine; $[Ca^{2+}]_i$, intracellular free calcium concentration; $[Ca^{2+}]_m$, mitochondrial free calcium concentration; $\Delta\psi$, mitochondrial membrane potential; PTC, proximal tubular cells; EGTA/AM, EGTA acetoxymethyl ester; AOAA, aminooxyacetic acid; DPPD, diphenylphenylenediamine; RR, ruthenium red; DCF-DA, 2′,7′-dichlorofluorescin diacetate; DCF, dichlorofluorescein; PI, propidium iodide.

ides; this strongly potentiates the dissipation of the mitochondrial membrane potential.

MATERIALS AND METHODS

Chemicals—EGTA/AM was from Molecular Probes, Inc. (Eugene, OR); collagenase (from *Clostridium histolyticum*), bovine serum albumin (Fraction V), digitonin, Fura-2, Fura-2/AM, rhodamine 123, EGTA, and Quin-2/AM were from Sigma; HEPES was from Boehringer Mannheim GmbH (Mannheim, Federal Republic of Germany); carboxymethoxylamine hemihydrochloride (aminooxyacetic acid (AOAA)), diphenylphenylenediamine (DPPD), and ruthenium red (RR) were from Aldrich Chemie (Brussels, Belgium); and 2',7'-dichlorofluorescin diacetate (DCF-DA) was from Serva GmbH (Heidelberg, FRG). DCVC was kindly provided by Dr. J. M. N. Commandeur (Department of Pharmacochemistry, Free University of Amsterdam, Amsterdam, The Netherlands).

Isolation of Proximal Tubular Kidney Cells—Male SPF Wistar/Wu rats from Sylvius Laboratories (Leiden, The Netherlands), weighing 200–250 g, were used throughout all the experiments. Animals were housed in Macrolon cages with hardwood bedding and had free access to food (MRH-B, Hope Farms B. V., Woerden, The Netherlands) and tap water. An alternating 12-h light and dark cycle was maintained in the animal rooms.

Proximal tubular kidney cells were isolated as described before in more detail (23), with one modification: prior to opening of the abdomen, rats were injected intravenously with 500 IU of heparin dissolved in 400 μl of saline. With this method, a cell preparation was routinely obtained that stained 95% positive for γ-glutamyltranspeptidase and 90% positive for nonspecific esterase and with a viability of 90–95% as determined by trypan blue exclusion (23). The cells were suspended in Hanks' balanced salt solution (137 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4 \cdot 7H_2O$, 0.4 mM $Na_2HPO_4 \cdot 2H_2O$, 0.4 mM KH_2PO_4, 1.3 mM $CaCl_2$, 4 mM $NaHCO_2$, 5 mM glycine, 20 mM HEPES), pH 7.4, containing 5 mM glucose and 2.0% (w/v) bovine serum albumin (Buffer A), gassed for 30 min with 95% O_2, 5% CO_2. For experiments in which PTC were incubated in nominally calcium-free buffer, PTC were washed three times with Hanks' balanced salt solution without $CaCl_2$ at 0 °C and thereafter were suspended in Hanks' balanced salt solution without $CaCl_2$.

Flow Cytometric Analysis of Hydroperoxide Formation, Δψ, and Cell Death in Proximal Tubular Cells in Suspension—Freshly isolated proximal tubular cells were suspended in Buffer A at a density of 0.5–1.0 × 10^6 cells/ml. The suspension routinely contained ~50% single cells, 45% cell clusters consisting of two to five cells, and ~5% cell clusters of up to 20 cells as determined by light microscopy. Cells were incubated in Costar culture flasks at 37 °C for 30 min prior to the start of the experiments.

Hydroperoxide formation was determined by flow cytometric analysis using the nonfluorescent probe DCF-DA. Release of the acetate moieties by intracellular esterase activity results in the release of dichlorofluorescin, which upon exposure to hydroperoxides, including both H_2O_2 and lipid peroxides, is hydrolyzed to the fluorescent probe dichlorofluorescein (DCF) (24). This probe has previously been used to detect the formation of hydroperoxides in several cell systems (10, 25–27). PTC were loaded with dichlorofluorescin by incubating them with 10 μM DCF-DA for 15 min at 37 °C, after which experiments were started. Prior to flow cytometric analysis, PI (5 μM final concentration) was added to the cells. The DCF and PI fluorescence properties of individual cells and cell clusters were analyzed using a FACScan flow cytometer (Becton Dickinson Advanced Cellular Biology, San Jose, CA) equipped with an argon laser using the Lysis program. DCF fluorescence was detected by the Fl_1 detector (emission at 500–550 nm corresponding to green fluorescence), and PI fluorescence was detected by the Fl_3 detector (emission at >600 nm corresponding to red fluorescence). The mean DCF fluorescence of PI-negative cells (*i.e.* still viable cells present in population R1 (Fig. 4*A*)) loaded with DCF-DA was calculated. For each separate experiment, the autofluorescence of PI-negative cells was determined. Hydroperoxide formation was expressed as the percentage of fluorescence at $t = 0$ and was calculated as follows: $((C - A)/(B - A)) \times 100\%$, where A is the autofluorescence of PI-negative cells, B is the DCF fluorescence of PI-negative cells at $t = 0$, and C is the DCF fluorescence of PI-negative cells from the sample. The method was validated by incubating PTC with 1.0 mM cumene hydroperoxide, which resulted in an increase in the green fluorescence of the cells in population R1, indicating the hydrolysis of dichlorofluorescin to DCF.

Δψ and cell death were determined by flow cytometry as described previously (19). Δψ was determined by analyzing the rhodamine 123 fluorescence intensity of PI-negative cells. Cell death was determined by counting the percentage of PI-positive cells. Δψ is expressed as the percentage of rhodamine 123 fluorescence at $t = 0$ (*i.e.* the start of the experiment) of those cells that stained PI-negative. Cell death is expressed as the percentage of PI-positive and rhodamine 123-negative cells.

Determination of $[Ca^{2+}]_i$ and Mitochondrial Free Calcium Concentration ($[Ca^{2+}]_m$)—Determination of free calcium concentrations using Fura-2 was essentially done as described previously (20, 28). Briefly, PTC were suspended in Buffer A containing 20 μM Fura-2/AM and allowed to adhere onto poly-D-lysine-coated coverslips for 1 h at 37 °C (28). The coverslips were mounted in a coverslip holder. Thereafter, cells were washed three times with Buffer A at 37 °C. The coverslip holder was placed on a temperature-controlled stage of the microscope, which kept the cell preparation at a temperature of 37 °C. An IM35 inverted microscope with a 50-watt mercury arc lamp (Zeiss, Oberkochen, FRG) equipped with a Nikon 40*\1.3 NA CF Fluor objective was used. Images were recorded using a CCD instrumentation camera controlled by a CC200 camera controller (Photometrics Ltd., Tucson, AZ). Images were processed on an Imagine image processing system (Synoptics, Cambridge, United Kingdom) and stored on the hard disk of a Hewlet-Packard 486 computer. From a group of the cells, the 470 nm emission images, after 340 and 380 nm excitation, were recorded for determination of the free intracellular calcium concentration. Determination of $[Ca^{2+}]_m$ was done essentially as described earlier by us and others (29, 30). The medium was replaced by mitochondrial buffer (250 mM sucrose, 20 mM KCl, 3 mM EGTA, 10 mM K_2HPO_4, 5 mM $MgCl_2$, 5 mM succinate) containing 100 μM digitonin (Buffer B). With this treatment, the plasma membrane became permeable within 30 s as evidenced by staining of the nucleus with propidium iodide and leakage of Fura-2 from the cells. Mitochondria remained intact since no change in the mitochondrial membrane potential was observed (as determined by rhodamine 123 staining). At 30 s after the addition of Buffer B, another 470 nm emission pair of images, after 340 and 380 nm excitation, of the same group of cells was recorded to determine mitochondrial calcium concentrations. After permeation of the plasma membrane, 15–20% of the total Fura-2 fluorescence was retained in the cell; it had the same localization as rhodamine 123, indicating that fluorescence was primarily located inside the mitochondria. This Fura-2 fluorescence was generally more than 5 times higher than the autofluorescence of nonloaded permeabilized control cells for both 340 and 380 nm excitation.

To investigate the effect of RR on $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$, PTC were incubated in Buffer A plus RR (30 μM). Prior to determination of $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$, the medium was replaced by Buffer A without RR. In a separate experiment, it was determined whether RR quenches the fluorescence of intracellular Fura-2. Cells were loaded with 20 μM Fura-2 for 60 min, followed by incubation for 45 min with 30 μM RR. Thereafter, cells were washed twice with Buffer A, and emission spectra of intracellular Fura-2 were recorded with a Perkin-Elmer LS-5B fluorometer. Preincubation of PTC with RR had no effect on the emission spectra for both 340 and 380 nm excitation.

Calcium concentrations were calculated essentially as described previously (20) and are expressed as the means ± S.E. of at least 60 individual cells, which were determined in at least five different cell isolations.

Statistics—Results are expressed as the means ± S.E. of four to five independent experiments, unless stated otherwise. Statistical significance between two groups was determined by means of an unpaired Student's t test. Statistical significant differences between groups were determined by means of a one-way analysis of variance.

RESULTS

Effect of DCVC on Hydroperoxide Formation—Hydroperoxide formation in PTC was investigated by flow cytometric analysis of PTC loaded with the hydroperoxide-sensitive fluorescent probe DCF-DA. The DCF and PI fluorescence properties of PTC were comparable to rhodamine 123 (Δψ) and PI staining of PTC (20). Three distinct populations of cells could be identified (Fig. 1*A*): population R1 with PI-negative fluorescence properties, indicating viable cells and cell clusters; population R2 with PI-positive fluorescence properties, indicating dead individual cells and cell clusters containing only dead cells; and population R3 with PI-positive fluorescence properties. Rhodamine 123 staining indicated that the latter population consists of cell clusters of both viable and dead cells (20).

Incubation of PTC with DCVC (400 μM) resulted in a signifi-

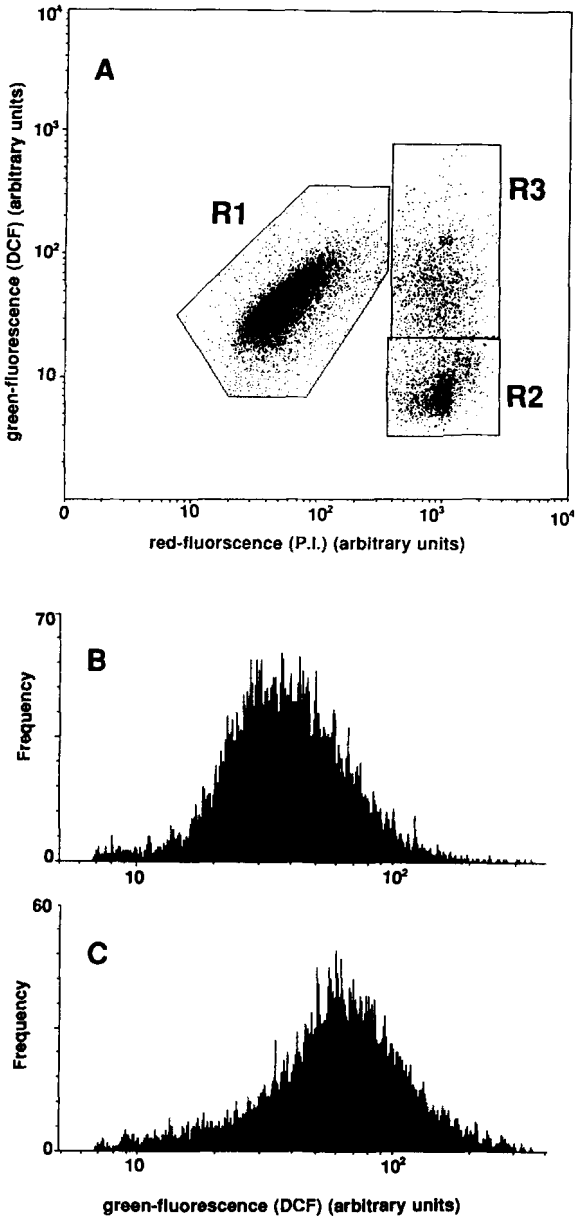

FIG. 1. **Flow cytometric analysis of hydroperoxide formation in freshly isolated proximal tubular cells.** PTC loaded with DCF-DA and propidium iodide were analyzed as described under "Materials and Methods." Shown is a two-dimensional density plot of green fluorescence (DCF) and red fluorescence (PI) of control cells (*A*). Three different populations can be identified as described under "Results." Also shown are the frequency histograms of the green fluorescence of DCF-positive and PI-negative cells (population R1) of control PTC (*B*) and PTC treated with 400 μM DCVC for 30 min (*C*). The difference between the control population (*B*) and the DCVC-treated population (*C*) was analyzed using the Kolmogorov-Smirnov test (Lysis II program of the FACScan system). The two populations had a 99% probability of difference.

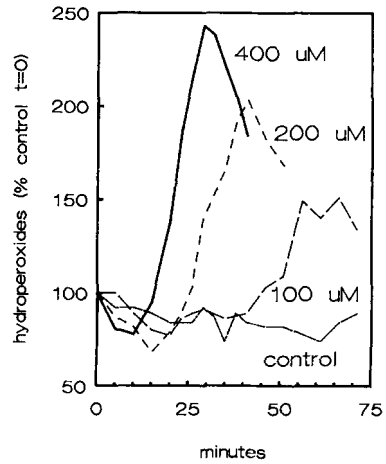

FIG. 2. **DCVC-induced hydroperoxide formation in freshly isolated PTC.** PTC were loaded and analyzed for dichlorofluorescein fluorescence as described for Fig. 1. At 15 min after the addition of DCF-DA, DCVC (100, 200, or 400 μM) was added. Every 3 min, a 100-μl sample was taken, added to a tube containing 5 μl of 100 μM propidium iodide, and directly analyzed by flow cytometry for DCF and PI fluorescence properties. Hydroperoxide formation was calculated as described under "Materials and Methods." Shown is one representative experiment of three.

TABLE I
Effect of various inhibitors on cumene hydroperoxide-induced cell death of PTC

The effect of cumene hydroperoxide on PTC viability was determined by flow cytometry as described for Fig. 1. AOAA (1 mM), EGTA/AM (20 μM), RR (30 μM), desferrioxamine (1 mM), and DPPD (20 μM) were added 15 min prior to the addition of cumene hydroperoxide (250 μM). Viability was determined after 30 min of incubation with cumene hydroperoxide. Shown is the percent cell death as means ± S.E. of three independent experiments.

	Cell death
	%
Control	22 ± 2
Cumene hydroperoxide	67 ± 3
+AOAA	63 ± 2
+EGTA/AM	62 ± 5
+RR	63 ± 3
+Desferrioxamine	27 ± 1
+DPPD	25 ± 2

cant increase in the DCF fluorescence of the propidium-negative cells (Fig. 1, *B* and *C*). The increase in hydroperoxide formation by DCVC over the control values was ~2.5-fold. It was both time- and concentration-dependent (Fig. 2) and occurred prior to the onset of cell death. At 400 μM DCVC, a peak in DCF fluorescence occurred after 30 min of incubation; subsequently, the fluorescence intensity of population R1 decreased (Fig. 2), which was due to cell death since there was a shift from cells from population R1 to the propidium-positive populations R2 and R3. The remaining cells in population R1 had a lower mean DCF fluorescence; these cells may have been more resistant to DCVC exposure.

To investigate whether pyridoxal phosphate-dependent β-lyase activity is required for DCVC-induced oxidative stress, PTC were pretreated for 15 min with AOAA, an inhibitor of pyridoxal phosphate-dependent enzymes. This pretreatment completely prevented the increase in hydroperoxide formation and cell death induced by 400 μM DCVC (data not shown). Inhibition of pyridoxal phosphate-dependent enzymes with AOAA did not affect the integrity of the PTC since the viability was unaffected compared with untreated PTC. The protective effect of AOAA was not due to antioxidant properties since preincubation of PTC with AOAA (1 mM) could not prevent cell death caused by the pro-oxidant cumene hydroperoxide (Table I).

The addition of the antioxidant DPPD (20 μM) to the incubation medium also completely prevented the formation of hydroperoxides (Fig. 3*A*). Their formation was also iron-dependent since chelation of iron with desferrioxamine (1 mM) resulted in a complete prevention of hydroperoxide formation (data not shown). DPPD still completely blocked oxidative stress when added 10 min after DCVC. When it was added after 15 or 20 min, it blocked a further increase in hydroperoxide formation (Fig. 3*A*).

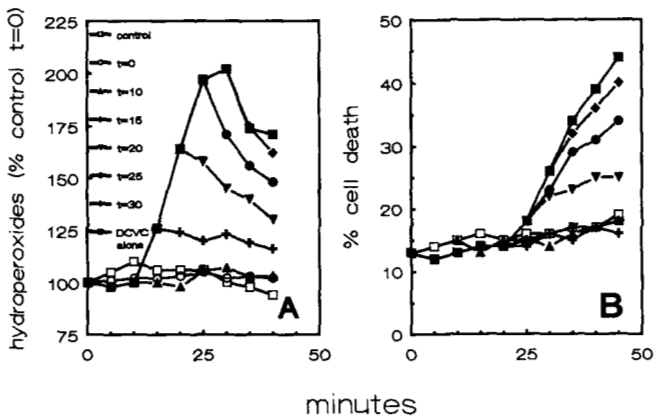

FIG. 3. **Effect of DPPD on DCVC-induced hydroperoxide formation and cell death.** PTC were analyzed for hydroperoxide formation (*A*) as described for Fig. 1. Cell death (*B*) was determined by analyzing the percentage of propidium iodide-positive cells. DPPD (20 μM) was added either simultaneously with or 10, 15, 20, 25, or 30 min after the addition of DCVC (400 μM). Shown is the mean of four to five independent experiments. Standard errors of the mean were <10% of the values, but are not shown for clarity.

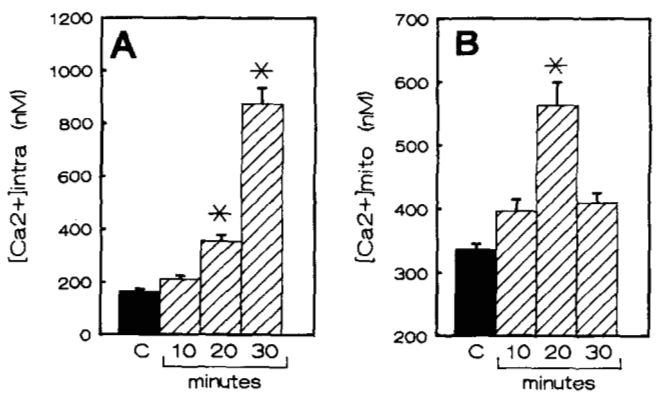

FIG. 4. **Effect of DCVC on $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$ in freshly isolated PTC.** Cellular and mitochondrial calcium concentrations were determined by video-intensified fluorescence microscopy as described under "Materials and Methods." Shown is the $[Ca^{2+}]_i$ (*A*) and $[Ca^{2+}]_m$ (*B*) of control cells and cells treated with 400 μM DCVC for 10, 20, and 30 min. *C* indicates control cells, which were analyzed between 0 and 30 min after the start of an experiment. No difference in either $[Ca^{2+}]_i$ or $[Ca^{2+}]_m$ during this time period was observed for these cells. Data are the means ± S.E. (n = 84–212 cells). *Asterisks* indicate significantly different from control ($p < 0.05$). Note the range on the *ordinate*.

TABLE II

Effect of RR on the DCVC-induced changes in $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$

The effect on $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$ was determined by video-intensified fluorescence microscopy as described for Fig. 3. RR (30 μM) was added 15 min prior to the addition of DCVC (400 μM); 20 min thereafter, $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$ were determined. Data are expressed as nanomolar calcium concentration. Shown are the means ± S.E. (n = 60–212 cells).

	$[Ca^{2+}]_i$	$[Ca^{2+}]_m$
Control	163 ± 11	336 ± 9
RR	189 ± 16	289 ± 16
DCVC	355 ± 23[a]	563 ± 37[a]
DCVC + RR	618 ± 117[a,b]	333 ± 24[b]

[a] Significantly different from treatment control ($p \leq 0.05$).
[b] Significantly different from DCVC only ($p \leq 0.05$).

DCVC-induced hydroperoxide formation occurred prior to cell death (Fig. 3*B*), which was also prevented by DPPD. The addition of DPPD after 15 min still effectively prevented cell death, whereas after 20 or 25 min, it delayed DCVC-induced cell death (Fig. 3*B*).

Effect of DCVC on $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$—DCVC induces an increase in $[Ca^{2+}]_i$ in freshly isolated PTC (20). In the present study, we investigated whether DCVC also affects mitochondrial calcium homeostasis. Therefore, the effect of DCVC on $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$ was determined in intact cells and in the same cells treated with digitonin, respectively, using the calcium sensitive-fluorescent probe Fura-2. Incubation of PTC with DCVC (400 μM) resulted in a time-dependent increase in $[Ca^{2+}]_i$ from 163 ± 11 nM for control cells to 874 ± 61 nM for cells treated with DCVC for 30 min (Fig. 4*A*). DCVC also induced a change in $[Ca^{2+}]_m$ measured in the same cells. A maximum increase in $[Ca^{2+}]_m$ was already observed after 20 min of incubation with DCVC; thereafter, $[Ca^{2+}]_m$ decreased again (Fig. 4*B*). To test whether this mitochondrial increase involves transport via the mitochondrial Ca^{2+} uniporter, PTC were incubated with DCVC together with an inhibitor of the uniporter, RR. RR (30 μM) did not prevent the increase in $[Ca^{2+}]_i$ induced by DCVC; however, it prevented an increase in $[Ca^{2+}]_m$ (Table II).

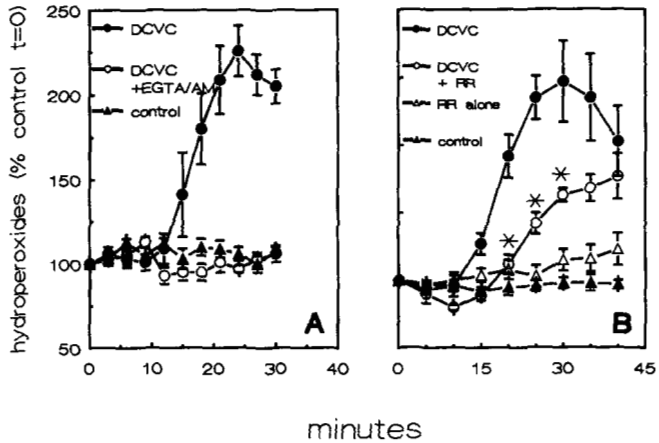

FIG. 5. **Effect of EGTA/AM (*A*) and RR (*B*) on DCVC-induced hydroperoxide formation.** PTC were analyzed for hydroperoxide formation as described for Figs. 1 and 2. At 15 min prior to the addition of DCVC (400 μM), EGTA/AM (20 μM) or RR (30 μM) was added to the cell suspension. Shown are the means ± S.E. of three separate experiments. *Asterisks* indicate significantly different from DCVC only ($p < 0.05$).

TABLE III

Effect of DPPD and desferrioxamine on the DCVC-induced changes in $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$

The effect on $[Ca^{2+}]$ was determined by video-intensified fluorescence microscopy as described under "Materials and Methods." Either DPPD (20 μM) or desferrioxamine (1 mM) was added together with DCVC (400 μM) to PTC; after 20 min of incubation, $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$ were determined. Data are expressed as nanomolar calcium concentration. Shown are the means ± S.E. (n = 60–77 cells).

	$[Ca^{2+}]_i$	$[Ca^{2+}]_m$
DPPD	213 ± 8	315 ± 18
+DCVC	431 ± 36[a]	456 ± 22[a]
Desferrioxamine	214 ± 18	310 ± 15
+DCVC	381 ± 26[a]	425 ± 18[a]

[a] Significantly different from treatment control ($p \leq 0.05$).

Role of Ca^{2+} in DCVC-induced Hydroperoxide Formation—Previously, we demonstrated that intracellular calcium perturbation is independent of extracellular calcium (20). To investigate whether omission of extracellular calcium affects hydroperoxide formation caused by DCVC, PTC were incubated in a nominally calcium-free buffer. This had no effect on DCVC-induced oxidative stress (data not shown), indicating that hydroperoxide formation is independent of extracellular calcium. We then investigated whether DCVC-induced hydroperoxide formation is dependent on changes in the cellular and mitochondrial calcium concentrations. To chelate intracel-

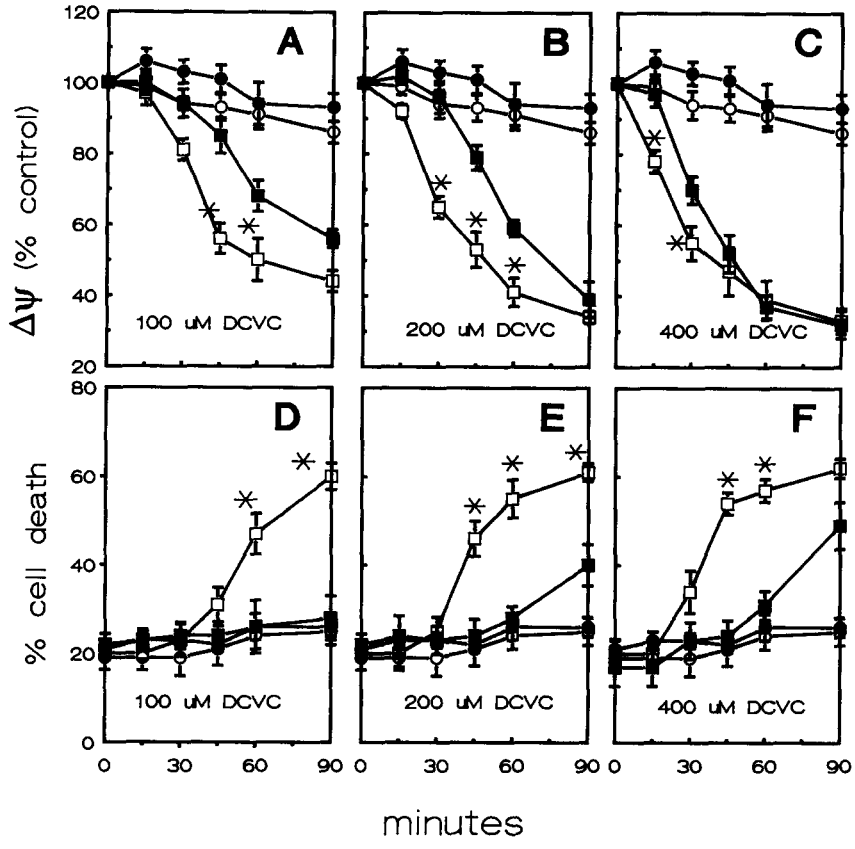

FIG. 6. **Effect of EGTA/AM on DCVC-induced dissipation of $\Delta\psi$ and cell death.** The effect on $\Delta\psi$ (*A–C*) and cell death (*D–F*) was analyzed by flow cytometric analysis of the rhodamine 123 and propidium iodide fluorescence properties of DCVC-treated PTC as described under "Materials and Methods." PTC were incubated with (*squares*) or without (*circles*) DCVC at a concentration of 100 μM (*A* and *D*), 200 μM (*B* and *E*), or 400 μM (*C* and *F*). EGTA/AM (*closed symbols*) was added 15 min prior to the addition of DCVC. Data are the means ± S.E. (n = 5). Note that the scale on the *y axis* ranges from 20 to 120%. *Asterisks* indicate significantly different from DCVC plus EGTA/AM ($p < 0.05$).

lular Ca^{2+}, PTC were incubated with DCVC together with EGTA/AM (20 μM). EGTA/AM completely prevented the increase in hydroperoxide formation (Fig. 5*A*). In addition, when PTC were pretreated with RR (30 μM) to prevent an increase in $[Ca^{2+}]_m$ without affecting the increase in $[Ca^{2+}]_i$ as described above, the formation of hydroperoxides was clearly reduced, but not completely prevented (Fig. 5*B*). Together, these data strongly suggest that oxidative stress is primarily dependent on an increase in mitochondrial Ca^{2+} concentrations.

To exclude the possibility that EGTA/AM provides protection by a desferrioxamine-like effect, we investigated whether cell death caused by the pro-oxidant cumene hydroperoxide can be prevented by EGTA/AM. Cumene hydroperoxide (250 μM) caused cell death of PTC within 30 min. This could be prevented by both desferrioxamine (1 mM) and DPPD (20 μM). In contrast, EGTA/AM (20 μM) did not prevent pro-oxidant-induced cell death (Table I). In addition, RR (30 μM) was also ineffective in protecting against cumene hydroperoxide-induced cell death (Table I). Thus, these data strongly indicate that the protection provided by both EGTA/AM and RR against DCVC-induced hydroperoxide formation is related to perturbations of intracellular calcium caused by DCVC.

We also tested the effect of DPPD and desferrioxamine on the DCVC-induced changes in $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$ by video-intensified fluorescence microscopy. Neither DPPD nor desferrioxamine prevented a DCVC-induced increase in $[Ca^{2+}]_i$ or $[Ca^{2+}]_m$ (Table III).

Role of Intracellular and Mitochondrial Ca^{2+} in DCVC-induced Dissipation of $\Delta\psi$ and Cell Death—The effect of DCVC on $\Delta\psi$ was followed by flow cytometric analysis of rhodamine 123 uptake by PTC. DCVC decreased $\Delta\psi$ in a time- and concentration-dependent way (Fig. 6, *A–C*), which was observed prior to cell death (Fig. 6, *D–F*). Chelation of cellular Ca^{2+} with EGTA/AM (20 μM) delayed the decrease in $\Delta\psi$ induced by 100, 200, or 400 μM DCVC (Fig. 6, *A–C*). Furthermore, EGTA/AM protected against cell death induced by these concentrations of DCVC (Fig. 6, *D–F*). However, a decrease in $\Delta\psi$ induced by 400 μM DCVC in the presence of EGTA/AM to ~40% of the control values was soon followed by cell death.

RR delayed the dissipation of $\Delta\psi$ induced by DCVC (400 μM) in a similar fashion compared with EGTA/AM (Fig. 7, *A* and *B*). Both compounds prevented DCVC-induced cell death (Fig. 8). DPPD and desferrioxamine also delayed the DCVC-induced dissipation of the mitochondrial membrane potential induced by 400 μM DCVC (Fig. 7, *C* and *D*).

DISCUSSION

In this study, we provide evidence that the Ca^{2+} concentration in the mitochondrial matrix is a critical factor for the induction of oxidative stress by the nephrotoxin DCVC. Furthermore, our data indicate that oxidative stress is involved in the dissipation of the mitochondrial membrane potential caused by DCVC (Fig. 9 and below).

Previously, we reported that DCVC increases $[Ca^{2+}]_i$ (20). Since studies in isolated heart mitochondria showed that incubation with high calcium concentrations results in an increased formation of ROS (9, 10), which could be prevented by an inhibitor of the mitochondrial Ca^{2+} uniporter, ruthenium red (10), we hypothesized that DCVC-induced oxidative stress, reported by others (20, 21), could be the result of changes in mitochondrial Ca^{2+} homeostasis. To test this hypothesis, we measured both hydroperoxide formation and the intracellular and mitochondrial free calcium concentrations in isolated rat PTC using flow cytometry and video-intensified fluorescence microscopy. DCVC clearly induced the formation of hydroperoxides in PTC prior to the onset of cell death. Furthermore, upon exposure of PTC to DCVC, the increase in $[Ca^{2+}]_i$ was accompanied by an increase in $[Ca^{2+}]_m$. The latter increase was dependent on the uptake of calcium via the mitochondrial uniporter since RR prevented this without affecting the elevation of $[Ca^{2+}]_i$. At later

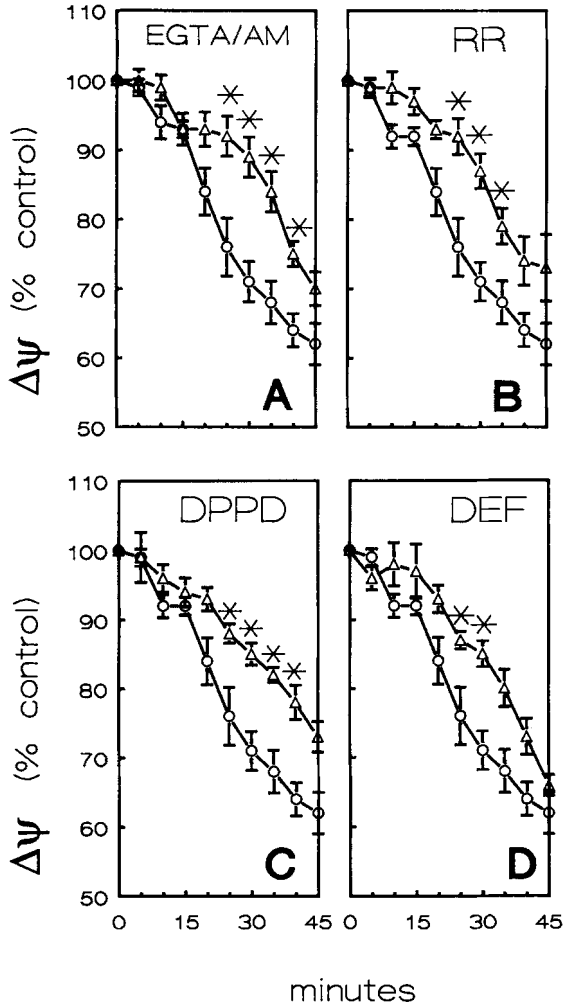

FIG. 7. **Effect of EGTA/AM (*A*), RR (*B*), DPPD (*C*), and desferrioxamine (*D*) on DCVC-induced decrease in Δψ.** The effect on Δψ was analyzed by flow cytometric analysis essentially as described under "Material and Methods." PTC (5–7 million/ml) were incubated with 1 μM rhodamine 123 for 15 min. Thereafter, the cell suspension was diluted such that the final rhodamine 123 concentration was 0.2 μM. PTC were pretreated with either EGTA/AM (20 μM) or RR (30 μM) for 15 min prior to the addition of DCVC (400 μM). DPPD (20 μM) and desferrioxamine (*DEF*; 1 mM) were added simultaneously with DCVC (400 μM). Propidium iodide (2 μM) was added just prior to the start of the experiments. Every 5 min, the rhodamine 123 fluorescence intensity of propidium iodide-negative cells was determined. Shown is the effect on Δψ expressed as a percentage of control. Incubation with RR, EGTA/AM, DPPD, or desferrioxamine alone had no effect on Δψ. ○, DCVC alone; △, DCVC with inhibitors. Values are the means ± S.E. of six independent experiments. *Asterisks* indicate significantly different from DCVC ($p < 0.05$). Note that the scale on the *ordinate* ranges from 50 to 120%.

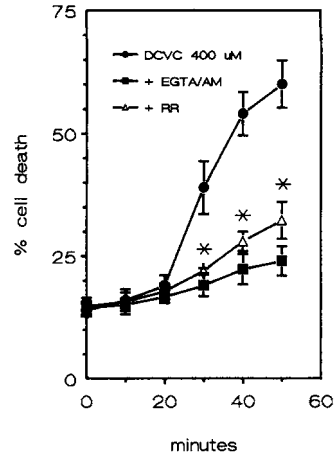

FIG. 8. **Effect of EGTA/AM and RR on DCVC-induced cell death.** The effect on cell death was determined by flow cytometric analysis of the percentage of propidium iodide-positive cells as described under "Materials and Methods." PTC were loaded with rhodamine 123 and propidium iodide as described for Fig. 7 and pretreated with either RR (30 μM) or EGTA/AM (20 μM) as described for Fig. 9. After the addition of DCVC (400 μM), the percentage of PTC that stained propidium iodide-positive and rhodamine 123-negative was determined every 10 min. Incubation with EGTA/AM or RR alone had no effect on cell viability. Shown are the means ± S.E. ($n = 5$). *Asterisks* indicate significantly different from DCVC only ($p < 0.05$).

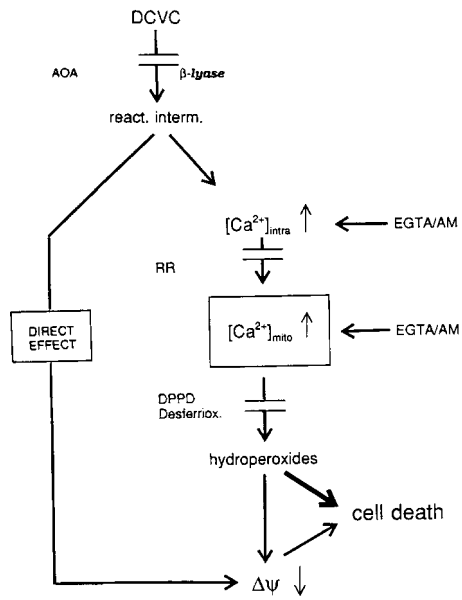

FIG. 9. **Scheme for proposed mechanism of dissipation of Δψ and cell death induced by DCVC.** For a description of the mechanism, see "Discussion."

time points, $[Ca^{2+}]_m$ decreased again, presumably due to the release of accumulated calcium from the mitochondrial matrix back into the cytosolic compartment or to precipitation of calcium phosphate in the mitochondria.

To determine whether DCVC-induced oxidative stress is dependent on an increase in $[Ca^{2+}]_i$ and/or $[Ca^{2+}]_m$, PTC were incubated with EGTA/AM and RR. Chelation of intracellular Ca^{2+} with EGTA/AM completely prevented the formation of hydroperoxides, whereas RR, which exclusively prevents the increase in $[Ca^{2+}]_m$, strongly delayed this effect. These data clearly indicate that increased intracellular Ca^{2+} concentration is involved in DCVC-induced hydroperoxide formation. Since RR had no effect on the DCVC-induced increase in $[Ca^{2+}]_i$ but provided appreciable protection against hydroperoxide formation, we suggest that the increase in $[Ca^{2+}]_m$ is the major factor in hydroperoxide formation. The difference between the effects of EGTA/AM and RR on the DCVC-induced formation of hydroperoxides is most likely due to a difference in the ability to lower $[Ca^{2+}]$ in the mitochondrial matrix: EGTA/AM will complex most of the free calcium present in the mitochondrial matrix, whereas RR only prevents additional uptake of Ca^{2+} in the mitochondria, leaving the Ca^{2+} already present in the mitochondrial matrix unaffected. Presumably, the higher the $[Ca^{2+}]_m$, the stronger the oxidative stress. DPPD and desferrioxamine, which both provided complete protection against the formation of hydroperoxides, did not have an effect on the DCVC-induced increase in $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$. This indicates that these calcium perturbations are not secondary to oxidative stress.

The formation of hydroperoxides caused by DCVC required

its metabolism by the pyridoxal phosphate-dependent renal cysteine-*S*-conjugate β-lyase since an inhibitor of this enzyme, AOAA, was completely protective. β-Lyase is present in high amounts in both the cytosol and the mitochondria of proximal tubular cells (33, 34). Upon β-cleavage, reactive metabolites are formed that bind covalently to macromolecules; this binding is completely prevented by AOAA (17–19, 32, 34). The mitochondrial localization of β-lyase activity corresponds with covalent binding and mitochondrial toxicity of DCVC (20, 32, 34, 35). Thus, our data are consistent with the current understanding (17–19, 32–35) that the irreversible cell injury caused by DCVC is dependent on cysteine-*S*-conjugate β-lyase activity.

Exposure of isolated mitochondria to either pro-oxidants (4–6, 13) or systems generating ROS (11, 12) leads to oxidative stress and subsequent mitochondrial damage. Indeed, antioxidants prevent a calcium-induced dissipation of $\Delta\psi$ (5–8). These findings prompted us to investigate a relationship between calcium-dependent oxidative stress and the dissipation of $\Delta\psi$ induced by DCVC. A time course showed that the initial rapid decrease in $\Delta\psi$ occurred immediately after the initiation of oxidative stress. This rapid decrease was delayed by inhibition of the formation of hydroperoxides with either DPPD or desferrioxamine. The extent of the delay was comparable to that observed with EGTA/AM or RR, which both prevented the drastic increase in hydroperoxide formation. These observations suggest that the DCVC-induced dissipation of $\Delta\psi$ is, at least in part, dependent on oxidative stress.

In addition to the above-described Ca^{2+}-dependent component, there was also a Ca^{2+}-independent component in the DCVC-induced decrease in $\Delta\psi$ since EGTA/AM delayed but did not prevent a $\Delta\psi$ decrease. The Ca^{2+}-independent effect on $\Delta\psi$ also requires DCVC bioactivation since the β-lyase inhibitor AOAA completely prevents it (20). This decrease is probably due to a direct inhibition of the respiratory chain, possibly by a DCVC-derived reactive metabolite, since incubation of isolated rat kidney mitochondria with DCVC decreases the oxygen consumption (31, 32), which is prevented by AOAA.

These data indicate that oxidative stress is an important event in DCVC-induced acute cell injury: inhibition of hydroperoxide formation protected against cell death, even when the antioxidant was added just prior to the onset of oxidative stress. Although inhibition of oxidative stress clearly protected against an initial rapid decrease in $\Delta\psi$ and cell death induced by DCVC, ultimately, a dissipation of $\Delta\psi$ was not prevented. Since this decrease was soon followed by cell death, we suggest that this cell killing is related to the Ca^{2+}-independent mitochondrial dysfunctioning induced by DCVC.

In conclusion, we propose the following mechanism for the dissipation of $\Delta\psi$ and cell death induced by DCVC (Fig. 9). Exposure of PTC to DCVC generates reactive intermediates due to DCVC metabolism by renal cysteine-*S*-conjugate β-lyase, resulting in a direct decrease in $\Delta\psi$. In addition, DCVC increases cytosolic calcium, which, in turn, leads to the uptake of calcium via the mitochondrial uniporter into the mitochondrial matrix. This increase in $[Ca^{2+}]_m$ induces, by an as yet unknown mechanism, the formation of hydroperoxides, which potentiates the above-described direct dissipation of $\Delta\psi$ by DCVC. Both oxidative stress and the dissipation of $\Delta\psi$ lead to lethal cell injury. These results suggest that $[Ca^{2+}]_m$ is an important factor in the regulation of hydroperoxide formation in intact cells and provide new insights in the relationship between mitochondrial calcium deregulation and oxidative stress in xenobiotic-induced mitochondrial injury.

REFERENCES

1. Carafoli, E. (1987) *Annu. Rev. Biochem.* **56,** 395–433
2. Gunter, T. E., and Pfeiffer, D. R. (1990) *Am. J. Physiol.* **258,** C755–C786
3. Nicholls, D., and Akerman, F. (1982) *Biochim. Biophys. Acta* **683,** 57–88
4. Richter, C., and Kass, G. E. N. (1991) *Chem. Biol. Intact.* **77,** 1–23
5. Novgorodov, S. A., Guda, T. I., Kushnareva, Y. E., Roginsky, V. A., and Kudrjashov, Y. B. (1991) *Biochim. Biophys. Acta.* **1058,** 242–248
6. Novgorodov, S. A., Gogvadze, V. G., Medvedev, B. I., and Zinchenko, V. P. (1989) *FEBS Lett.* **248,** 179–181
7. Novgorodov, S. A., Gudz, T. I., Mohr, Y. E., Goncharenko, E. N., and Yaguzhinsky, L. S. (1989) *FEBS Lett.* **247,** 255–258
8. Carbonera, D., and Azzone, G. F. (1988) *Biochim. Biophys. Acta* **943,** 245–255
9. Cadenas, E., and Boveris, A. (1980) *Biochem. J.* **188,** 31–37
10. Chacon, E., and Acosta, D. (1991) *Toxicol. Appl. Pharmacol.* **107,** 117–128
11. Malis, C. D., and Bonventre, J. V. (1986) *J. Biol. Chem.* **261,** 14201–14208
12. Harris, E. J., Booth, R., and Cooper, M. B. (1982) *FEBS Lett.* **146,** 267–272
13. Richter, C., and Frei, B. (1988) *Free Radical Biol. & Med.* **4,** 365–375
14. Lock, E. A. (1988) *CRC Crit. Rev. Toxicol.* **19,** 23–42
15. Lock, E. A. (1989) *Toxicol. Lett.* **46,** 93–106
16. Nagelkerke, J. F., and Boogaard, P. J. (1991) *Life Sci.* **49,** 1769–1776
17. Dekant, W., Vamvakas, S., and Anders, M. W. (1989) *Drug Metab. Rev.* **20,** 43–83
18. Commandeur, J. M. N., and Vermeulen, N. P. E. (1990) *Chem. Res. Toxicol.* **3,** 212–218
19. Chen, Q., Jones, T. W., Brown, P. C., and Stevens, J. L. (1990) *J. Biol. Chem.* **265,** 21603–21611
20. van de Water, B., Zoeteweij, J. P., de Bont, H. G. J. M., Mulder, G. J., and Nagelkerke, J. F. (1993) *Biochem. Pharmacol.* **45,** 2259–2267
21. Groves, C. E., Lock, E. A., and Schnellmann, R. G. (1991) *Toxicol. Appl. Pharmacol.* **107,** 54–62
22. van de Water, B., Zoeteweij, J. P., de Bont, H. G. J. M., Mulder, G. J., and Nagelkerke, J. F. (1992) *Toxicol. Lett.* (suppl.) 209
23. Boogaard, P. J., Mulder, G. J., and Nagelkerke, J. F. (1989) *Toxicol. Appl. Pharmacol.* **101,** 135–143
24. Cathcart, R., Schwiers, E., and Ames, B. N. (1983) *Anal. Biochem.* **134,** 111–116
25. Himmelfarb, J., Hakim, R. M., Holbrook, D. G., Leeber, D. A., and Ault, K. E. (1992) *Cytometry* **13,** 83–89
26. Scott, J. A., Homcy, C. J., Khaw, B. A., and Rabito, C. A. (1988) *Free Radical Biol. & Med.* **4,** 79–83
27. Burow, S., and Valet, G. (1987) *Eur. J. Cell Biol.* **43,** 128–133
28. Zoeteweij, J. P., van de Water, B., de Bont, H. J. G. M., Mulder, G. J., and Nagelkerke, J. F. (1992) *Biochem. J.* **288,** 207–213
29. Zoeteweij, J. P., van de Water, B., de Bont, H. J. G. M., Mulder, G. J., and Nagelkerke, J. F. (1993) *J. Biol. Chem.* **268,** 3384–3388
30. Chacon, E., Ulrich, R., and Acosta, D. (1992) *Biochem. J.* **281,** 871–878
31. Lash, L. H., and Anders, M. W. (1987) *Mol. Pharmacol.* **32,** 549–556
32. Hayden, P. J., and Stevens, J. L. (1990) *Mol. Pharmacol.* **37,** 468–476
33. Stevens, J. L., Robbins, J. D., and Byrd, R. A. (1986) *J. Biol. Chem.* **261,** 15529–15537
34. Stevens, J. L., Ayoubi, N., and Robbins, J. D. (1988) *J. Biol. Chem.* **263,** 3395–3401
35. Elfarra, A. A., Lash, L. H., and Anders, M. W. (1986) *Mol. Pharmacol.* **31,** 208–212