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THE RELATIONSHIP BETWEEN INTRACELLULAR Ca^{2+} AND THE MITOCHONDRIAL MEMBRANE POTENTIAL IN ISOLATED PROXIMAL TUBULAR CELLS FROM RAT KIDNEY EXPOSED TO THE NEPHROTOXIN 1,2-DICHLOROVINYL-CYSTEINE

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Abstract—The effects of 1,2-dichlorovinyl-cysteine (DCVC) on the intracellular free calcium concentration ($[Ca^{2+}]_i$) and the mitochondrial membrane potential ($\Delta\Psi$) were investigated in freshly isolated rat kidney proximal tubular cells (PTC). Prior to cell death, DCVC induced a rise in $[Ca^{2+}]_i$ and a decrease in the $\Delta\Psi$. Omission of extracellular calcium still resulted in a DCVC-induced increase of $[Ca^{2+}]_i$, indicating that calcium was released from intracellular stores. The β-lyase inhibitor amino-oxyacetic acid completely protected against mitochondrial damage and cell death, indicating that the DCVC effects are dependent on β-lyase metabolism. Incubation of the PTC with DCVC together with the intracellular-calcium complexing agents EDTA/acetoxy-methyl (AM), EGTA/AM or Quin-2/AM delayed (but did not prevent) the decrease of the $\Delta\Psi$ and cell death, which indicates a relationship between $[Ca^{2+}]_i$ and the decrease of $\Delta\Psi$. In individual cells four different responses induced by DCVC were observed; an increase of $[Ca^{2+}]_i$ without an effect on $\Delta\Psi$, a decrease of $\Delta\Psi$ and an increase of $[Ca^{2+}]_i$ occurring simultaneously; an increase of $[Ca^{2+}]_i$ preceded by a decrease of $\Delta\Psi$ and a decrease of $\Delta\Psi$ without any increase of $[Ca^{2+}]_i$. This indicates that the DCVC-induced effects on $[Ca^{2+}]_i$ and $\Delta\Psi$ can appear independently. The data show that mitochondrial damage is potentiated by an elevation of $[Ca^{2+}]_i$, thereby creating a situation which rapidly leads to cell death.

Mitochondria play an important role in the regulation of intracellular calcium homeostasis [1, 2]. In pathological conditions an accumulation of calcium in the mitochondria has been found in necrotic tissue. This led to the idea that calcium could be involved in mitochondrial damage, finally resulting in cell death. The mechanisms by which calcium induces mitochondrial injury have been extensively investigated in isolated mitochondria [2–4]. Several investigations indicate that an extensive increase in cellular calcium levels induce mitochondrial damage [5, 6]. However, the role of calcium in xenobiotic-induced mitochondrial damage in intact living cells is less well defined.

1,2-Dichlorovinyl-cysteine (DCVC†), a cysteine conjugate of trichloroethylene, belongs to the group of nephrotoxic haloalkenyl-*S*-cysteine conjugates. DCVC induces severe proximal tubular necrosis in various mammalian species [7–9]. Although many studies have been done to elucidate the mechanism of toxicity of DCVC and related compounds [10–13], the exact pathway leading to cell death is still unclear. The nephrotoxicity of DCVC and related halogenated alkenes is believed to be directed mainly towards the mitochondria of proximal tubular cells (PTC), and is dependent on metabolism by the enzyme β-lyase (EC 4.4.1.13). Mitochondrial damage has been demonstrated by a reduced cellular oxygen consumption in isolated PTC, resulting in depletion of cellular ATP [14, 15], by loss of the ability of isolated rat kidney mitochondria to sequester calcium [16] and by inhibition of state 3 and state 4 mitochondrial respiration [17]. Whether mitochondrial damage is the only and critical step leading to irreversible cell injury is still a point of debate. Recent work shows that DCVC increased intracellular calcium in the LLC-PK1 cell line after 24 hr [18]. However, DCVC had hardly any effect on intracellular free calcium concentration ($[Ca^{2+}]_i$) in isolated proximal tubules isolated from rabbit kidney, at least not after 5 min incubation [19]. An elevated calcium level was associated with DCVC cytotoxicity in the LLC-PK1 cell line, since cytotoxicity was prevented by buffering free intracellular calcium by the acetoxymethyl esters of the calcium chelators Quin-2 and EGTA [20]. These data suggest that besides mitochondrial damage, calcium also plays a role in haloalkene induced cytotoxicity. It is however still unclear whether a DCVC-induced rise in free intracellular calcium also occurs in normal rat PTC and whether such an

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† Abbreviations: $[Ca^{2+}]_i$, intracellular free calcium concentration; $\Delta\Psi$, mitochondrial membrane potential; DCVC, 1,2-*S*-dichlorovinyl-L-cysteine; VIFM, video intensified fluorescence microscopy; PTC, proximal tubular cells; BSA, bovine serum albumin; HBSS, Hank's balanced salt solution; AM, acetoxy-methyl.

increase is related to DCVC-induced mitochondrial damage and cell death.

The aim of this study was to investigate in individual living PTCs the effect of DCVC on $[Ca^{2+}]_i$ and on the mitochondrial membrane potential ($\Delta\Psi$) in relation to cell death. To this purpose flow cytometry and video intensified fluorescence microscopy (VIFM) were used. With the latter method it was possible to study the relationship between DCVC-induced effects on the cellular calcium homeostasis and the $\Delta\Psi$ in individual cells.

MATERIALS AND METHODS

Chemicals. Bovine calf serum (BCS) (Hyclone Laboratories, Logan, UT, U.S.A.), penicillin (Gist-Brocades, Delft, The Netherlands), ciprofloxacin (Bayer A.G., Leverkusen, F.R.G.), amphotericine B (Squibb, Rijswijk, The Netherlands), EDTA/acetoxy-methyl (AM), EGTA/AM (Molecular Probes, Eugene, OR, U.S.A.), minimum essential D-valine medium, concentrated vitamin solution, concentrated non-essential amino acid solution, propidium iodide, collagenase from *Clostridium histolyticum*, bovine serum albumin fraction V (BSA), FURA-2, FURA-2/AM, rhodamine-123, EGTA, Quin-2/AM (the Sigma Chemical Co., St Louis, MO, U.S.A.), HEPES (Boehringer Mannheim, GmbH, Mannheim, F.R.G.), carboxymethoxylamine hemihydrochloride (amino-oxyacetic acid, AOAA) (Aldrich-Chemie, Brussels, Belgium), heparin (Organon-Teknica B.V., Boxtel, The Netherlands). DCVC was kindly provided by Dr J. M. N. Commandeur (Department of Pharmacochemistry, Free University of Amsterdam, The Netherlands).

Animals. Male SPF Wistar/Wu rats from the Sylvius Laboratories (Leiden, The Netherlands), weighing 200–250 g, were used throughout all the experiments. Rats were fed regular chow (Hope Farms B.V., Woerden, The Netherlands) and tap water *ad lib.*

Isolation of proximal tubular kidney cells. Proximal tubular kidney cells were isolated as described before in more detail [21], with one modification: prior to opening of the abdomen, rats were injected i.v. with 500 IU heparin dissolved in 400 μL saline. Routinely a preparation of cells which stained 95% positive for γ-glutamyl-transpeptidase and 90% positive for non-specific-esterase was obtained [21].

Flow cytometric analysis of the ($\Delta\Psi$) and cell death in PTCs in suspension. Freshly isolated PTCs were suspended in Hank's balanced salt solution (HBSS) (pH 7.4, in mM, 137 NaCl, 5 KCl, 0.8 $MgSO_4.7H_2O$, 0.4 $Na_2HPO_4.2H_2O$, 0.4 KH_2PO_4, 1.3 $CaCl_2$, 4 $NaHCO_2$, 20 HEPES) containing 5 mM glucose and 0.2% (w/v) BSA (Buffer A), at a density of 0.5–1.0 × 10^6 cells/mL. The cell suspension routinely consisted of about 50% of single cells, 45% cell-clusters of two to five cells and about 5% of cell-clusters of up to 30 cells. Cells were incubated in Costar culture flasks at 37° under 95% O_2/5% CO_2. After 30 min preincubation, DCVC either with or without EDTA/AM, EGTA/AM or Quin-2/AM was added to the incubation flasks. $\Delta\Psi$ and viability were determined at 0, 15, 45, 90, 135 and 180 min after addition of the compounds.

$\Delta\Psi$ and viability were determined by analysing the rhodamine-123 [22, 23] and propidium iodide fluorescence properties of the cells respectively, with a FACScan flow cytometer (Becton Dickenson, San Jose, CA, U.S.A.) equipped with an Argon laser, using the Lysis program (Becton Dickenson). Cells were loaded with rhodamine-123 as follows. A 500 μL sample of the cell suspension was added to 100 μL of 6 μM rhodamine-123 solution in Buffer A. After 15 min equilibration with rhodamine-123 at 37°, the suspension was centrifuged for 1 min at 80 *g*. The pellet was resuspended in 250 μL of Buffer A, 37°, containing 0.2 μM rhodamine-123 and 2 μM propidium iodide, to prevent loss of fluorescent label and to stain non-viable cells respectively. Analysis was done using a flow rate of 12 μL/min. Rhodamine-123 fluorescence was detected by the Fl_1-detector, with a emission detection below 560 nm. Propidium iodide fluorescence was detected by the Fl_3-detector, with emission detection above 620 nm. Per sample 2000–5000 cells were counted. The method was validated by incubation of PTC with potassium cyanide previously shown to decrease the $\Delta\Psi$ [24].

Shown in Fig. 2 is the time course of the change in $\Delta\Psi$ of the cells present in R1, expressed as the percentage of $\Delta\Psi$ of the cells in R1 at $T = 0$; and the time course of the change in the percentage of total cells in R1. Viability was expressed as the percentage of propidium iodide positive cells in population R2 (% cell death) and corresponded with Trypan blue uptake.

Determination of free intracellular $[Ca^{2+}]_i$ by Quin-2 fluorimetry. Intracellular free Ca^{2+} in freshly isolated PTC was determined as described previously [25]. At various time points samples of 1.5 mL were taken from a suspension of PTC in HBSS containing 5 mM glucose and 2% BSA (Buffer B) (2.0–3.0 × 10^6 cells/mL). After centrifugation for 1 min at 80 *g*, 1 mL of Buffer B was added together with 5 μL of a 10 mM Quin-2/AM stock in dimethyl sulphoxide. After 10 min incubation at 37° the suspension was centrifuged for 5 sec at 500 *g*, and the pelleted cells were resuspended in 3 mL of Buffer A, and transferred to a cuvette, which was kept at 37°, in a Perkin–Elmer LS-5 fluorimeter; excitation wavelength was 339 nm, and emission wavelength 492 nm. Fluorescence signal was allowed to stabilize for 1 min before addition of 3 μL of a solution of 5 mM digitonin to determine maximum fluorescence, followed by addition of 100 μL of 500 mM EGTA to determine minimum fluorescence. $[Ca^{2+}]_i$ (nM) was calculated by the following equation:

$$(F - F_{min}/F_{max} - F) \times K_d$$

where F is fluorescence signal of the Quin-2 loaded cell suspension, F_{max} the fluorescence signal after addition of digitonin and F_{min} the fluorescence signal after addition of EGTA. The value of K_d was determined by constructing a Quin-2 calibration curve and was 115 nM.

Loading of the cells with FURA-2/AM and rhodamine-123. Isolated rat kidney PTCs were suspended in minimum essential D-valine medium, supplemented with 100 IU penicillin/L, 4.0 mg

ciprofloxazin/L, 2.5 mg amphotericin/L, 0.1% (w/v) BSA, 1.0% (v/v) concentrated vitamin solution, 1.0% (v/v) concentrated non-essential amino acids solution, 1 mM sodium pyruvate, 2 mM L-glutamine and 10% (v/v) bovine calf serum. Cells were plated at a density of 0.1–0.2 × 10^6 cells/cm^2, in six well culture dishes containing glass coverslips coated with rat tail collagen. Cells were allowed to adhere for at least 3 hr before starting the experiments. During this period primarily cell-clusters adhered, consisting of 5–20 cells. Experiments with adhered cells were started between 3 and 10 hr after isolation. Cells attached on collagen coated coverslips were incubated in Buffer B, supplemented with 10 μM FURA-2/AM and 1 μM rhodamine-123, for 30–40 min at 37° in a 95% air/5% CO_2 humidified atmosphere. Thereafter, cells were washed three times with Buffer B. Coverslips with the loaded PTCs were mounted on a coverslip holder, and Buffer B containing 0.2 μM rhodamine-123 (to prevent loss of label) and 1 μM propidium iodide was added on top of the coverslip. Loading with FURA-2/AM resulted in a diffuse staining of the cells, whereas rhodamine-123 specifically stained the mitochondria.

Determination of $[Ca^{2+}]_i$, $\Delta\Psi$ *and cell death.* The coverslip holder was placed on a thermostated microscope table which kept the bath solution at a temperature of 37°. An IM35 inverted microscope with a 50 W mercury arc lamp (Zeiss, Oberkochen, F.R.G.) and a Nikon 40 × 1.4 NA CF Fluor lens was used. Images were recorded using a CCD instrumentation camera, controlled by a CC200 camera controller (Photometrics, Tucson, AZ, U.S.A.). Images were processed on an Imagine image processing system (Synoptics, Cambridge, U.K.) and stored on the hard disk of a Hewlett Packard 486 computer.

$[Ca^{2+}]_i$ were determined by the digital ratio imaging method [26]. Cells, loaded with FURA-2 were excitated at 340 nm or 380 nm for calcium-dependent and calcium-independent FURA-2 fluorescence determination respectively. A dichroic mirror of 395 nm and a 470 nm longpass emission filter were used. Images were recorded with the CCD camera in full frame mode. Exposure times for 340 nm fluorescence measurements were always 2-fold longer than for 380 nm, and varied between 200 and 1000 msec depending on the degree of FURA-2 loading of the cells. Images were corrected for background by means of background subtraction before calculation of the ratio images. Ratio (R) images, for R = 340 nm/380 nm, were calculated by dividing fluorescence intensity of 340 nm images by fluorescence intensity of 380 nm images on a pixel to pixel basis. For calibration, pixel fluorescence intensities were recorded at both 340 nm and 380 nm, of HBSS (pH 7.4, 37°) containing HEPES (20 mM), EGTA (2 mM), FURA-2 (20 μM), with or without $CaCl_2$ (10 mM) for saturating calcium and no calcium conditions respectively. Sucrose [60% (w/v)] was added to correct for higher viscosity of the intracellular compartment compared to the media, as has been described earlier [27]. $[Ca^{2+}]_i$ were calculated by the following equation:

$$[Ca^{2+}]_i = K_d\beta[(R - R_{min})/(R_{max} - R)]$$

with K_d = 224 nM as the equilibrium dissociation constant for Ca^{2+} and FURA-2 [28]; $R_{min} = (F_{340}/F_{380})$ at no calcium, $R_{max} = (F_{340}/F_{380})$ at saturating calcium (10 mM) and $\beta = F_{380}$ (no calcium)/F_{380} (saturated calcium), where F is the pixel fluorescence intensity.

$\Delta\Psi$ was determined by measuring the relative fluorescence emission intensity of rhodamine-123 loaded cells. For excitation and emission a 450–490 bandpass filter, a 510 nm dichroic mirror and a 520 longpass filter were used. Exposure times varied from 20 to 80 msec depending on the degree of rhodamine-123 loading. Images were corrected for background fluorescence before determination of the fluorescence intensities of the cells. Relative fluorescence emission intensities were determined for each individual cell by summation of all individual pixel intensities of a cell. $\Delta\Psi$ was expressed as the percentage relative fluorescence emission intensity of the control timepoint t = 0 min. The $[Ca^{2+}]_i$ and $\Delta\Psi$ were determinated in the same cell.

Dead cells were detected by the intense staining of the cell nucleus with propidium iodide. For excitation and emission a 510–560 bandpass filter, a 580 nm dichroic mirror and a 590 longpass filter were used.

Statistics. All experiments were done five times. Results are expressed as the means ± SEM of five independent experiments, unless otherwise stated. Statistical analysis was done by means of an unpaired Student's t-test or one-way analysis of variance (ANOVA).

RESULTS

Role of $[Ca^{2+}]_i$ *in DCVC-induced toxicity in freshly isolated PTC as detected by flow cytometry*

The effect of DCVC on the ($\Delta\Psi$) of freshly isolated PTC was investigated by flow cytometric analysis of PTC loaded with rhodamine-123, which enabled studies on the DCVC-induced effects in a large population of cells.

Three distinct cell populations could be identified according to rhodamine-123 and propidium iodide-staining properties of the cells (Fig. 1). Population R1 with a high rhodamine-123 fluorescence and low propidium iodide fluorescence, indicating the presence of viable single cells and cell-clusters consisting of only viable cells. Population R2 with a low mean rhodamine-123 fluorescence and high propidium iodide fluorescence, representing non-viable single cells and cell-clusters containing only non-viable cells. Population R3 with a high rhodamine-123 fluorescence as well as a high propidium iodide fluorescence, indicating the presence of cell-clusters consisting of both viable and non-viable cells. Upon treatment with 50 μM digitonin populations R1 and R3 disappeared, while population R2 increased correspondingly.

During incubation of PTC for 45 min with DCVC (100 μM) the mean rhodamine fluorescence intensity of population R1 decreased, indicating a decrease in the mean $\Delta\Psi$ (Fig. 2A). After this period the percentage of the cells in population R1 decreased (Fig. 2B), which corresponded with an increase in population R2, indicating cell death (Fig. 2C). The

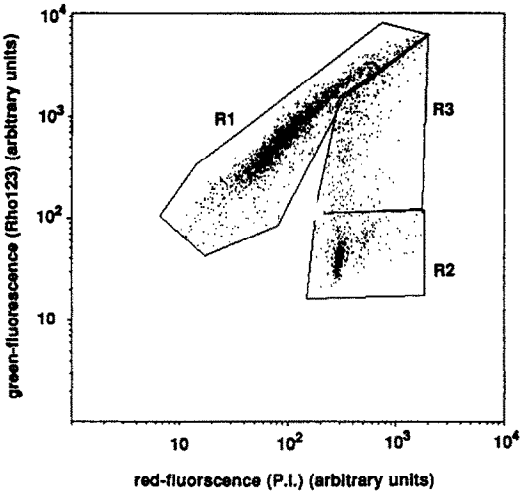

Fig. 1. Flow cytometric analysis of the $\Delta\Psi$ of PTC. Freshly isolated PTCs were loaded with rhodamine-123. The data obtained for red-fluorescence (propidium iodide) (X-axis) and green-fluorescence (rhodamine-123) (Y-axis) analysis of 10,000 particles are shown. Three distinct populations are present (as indicated by the drawn line): population R1 with a high rhodamine-123 (Rho123) and low propidium iodide (P.I.) fluorescence intensity, population R2 with a low Rho123 and high P.I. fluorescence intensity and population R3 with high Rho123 and high P.I. fluorescence.

percentage of cells in population R3 ranged from 9 to 14% and did not change significantly in either control or DCVC treated cells during the 3-hr incubation period. The effects induced by DCVC could be prevented completely by the β-lyase inhibitor amino-oxyacetic acid (AOAA) (1 mM) (Table 1).

The effect of DCVC on the cellular calcium homeostasis was investigated by determination of $[Ca^{2+}]_i$ in freshly isolated PTC. To get a sufficient Quin-2 fluorescence signal, PTCs were incubated at a four times higher density than used for the studies described above. Incubation of freshly isolated PTC with DCVC (100 μM) resulted in a 5-fold increase of $[Ca^{2+}]_i$ (Fig. 3A). $\Delta\Psi$ and cell death were determined for the same cells by flow cytometry. Although the above mentioned data show that the $\Delta\Psi$ was decreased after 45 min incubation with DCVC (100 μM), the $\Delta\Psi$ of PTC incubated at higher density but exposed to the same concentration of DCVC, was still intact after 40 min and started to decrease after 80 min (Fig. 3B). Before this timepoint no increase in cell death was observed (Fig. 3C). These data indicate that the increase of $[Ca^{2+}]_i$ occurred prior to a decrease of $\Delta\Psi$ and cell death.

To investigate whether this increase of intracellular free calcium plays a role in the DCVC-induced decrease of the $\Delta\Psi$, the effect of the cell-permeable acetoxy-methyl ester of EDTA, EDTA/AM, was tested with freshly isolated PTC incubated with DCVC (100 μM). This compound can readily enter the cell and complex intracellular free calcium. EDTA/AM (10 μM) prevented cell death from

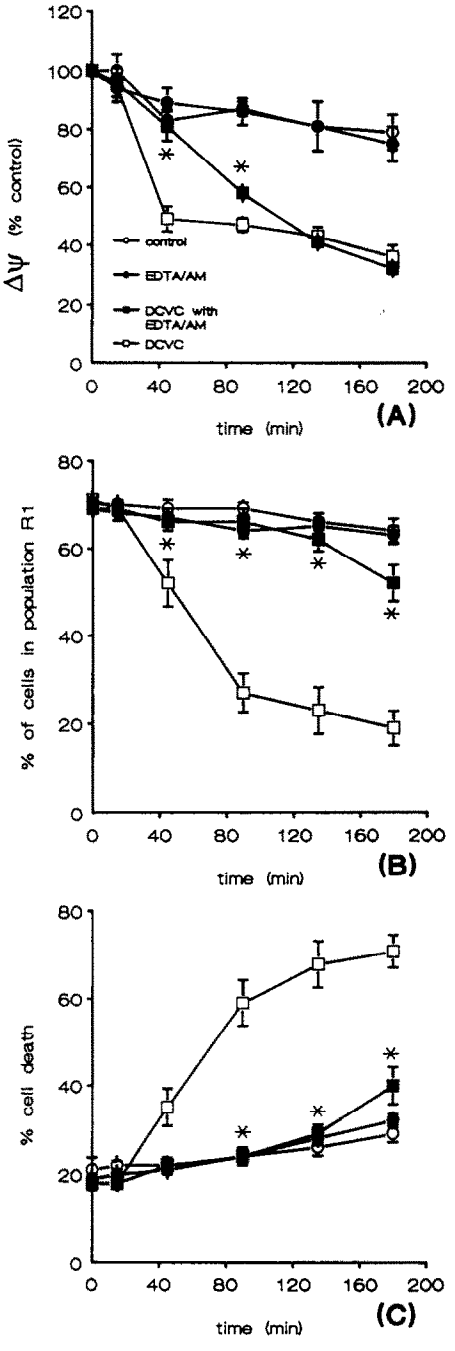

Fig. 2. Effect of EDTA/AM on the DCVC-induced decrease of $\Delta\Psi$ of freshly isolated PTC as determined by flow cytometric analysis. Cells were incubated with (squares) or without (circles) 100 μM DCVC, together with (closed symbols) or without (open symbols) addition of 10 μM EDTA/AM. Rhodamine-123 and propidium iodide fluorescence properties of 2000–5000 particles were analysed as described in the Materials and Methods. Viability of PTC was determined as the per cent of propidium positive cells. The mean relative green-fluorescence intensities of population R1 (A), the % of cells in population R1 (B) and % cell death (C) at different timepoints are shown. Results shown are the mean ± SEM of five independent experiments. Asterisk indicates significantly different from DCVC treatment alone (ANOVA, $P < 0.05$).

Table 1. Effect of amino-oxyacetic acid (AOAA) on DCVC-induced decrease of $\Delta\Psi$ in freshly isolated PTC

	$\Delta\Psi$	% of cells in population R1	% cell death
Control	79 ± 6	64 ± 3	27 ± 3
+ 100 μM 1,2-DCVC	36 ± 4*	19 ± 4*	71 ± 4*
1 mM AOAA	73 ± 6	65 ± 3	27 ± 2
+ 100 μM 1,2-DCVC	74 ± 5	63 ± 3	28 ± 3

Cells were treated as described in the Materials and Methods. AOAA (1 mM) was added together with 1,2-DCVC.

Shown are the mean ± SEM of $\Delta\Psi$, % of cells in population R1 and % cell death after 180 min incubation (N = 5).

* Indicates significantly different from control (ANOVA, $P < 0.05$).

occurring within the 3-hr incubation (Fig. 2C); it delayed but did not prevent the DCVC-induced decrease of $\Delta\Psi$ (Fig. 2A). EDTA/AM (10 μM) alone had no effect on $\Delta\Psi$ and did not cause cell death (Fig. 2A and C). The tetra-AM esters of two other calcium chelators, EGTA/AM (20 μM) and Quin-2/AM (10 μM), had similar effects as EDTA/AM (results not shown). These results indicate that intracellular free calcium plays a role in the early decrease of the $\Delta\Psi$ induced by DCVC in PTCs.

Effects of DCVC on $[Ca^{2+}]_i$ and $\Delta\Psi$ as detected by VIFM

The DCVC-induced effects on the cellular calcium homeostasis and $\Delta\Psi$ were investigated in more detail by VIFM. In these studies freshly isolated cells were used which were allowed to adhere onto collagen-coated glass coverslips. During an incubation period of at least 3 hr before experiments were started, cells and cell-clusters attached and spread onto the collagen-coated coverslips.

After this atttachment period, DCVC (0.5 mM) was added. Within 50 min cells which had spread retracted from their collagen substrate and rounded up. In approx. 10% of the cells, blebbing occurred after 90 min of incubation. No change in morphology was observed in control cells.

The $[Ca^{2+}]_i$ in resting cells was 162 ± 16 nM (mean ± SEM, N = 30). It varied between cells within a cell-cluster (from 32 nM to 280 nM). $[Ca^{2+}]_i$ did not change in control incubations during the 90-min incubation period, the addition of 0.5 mM DCVC resulted in an increase. This increase started after about 50 min and reached a maximum of 395 ± 26 nM after 80 min (not shown). Variation of the DCVC-induced increase of $[Ca^{2+}]_i$ between individual cells could be observed within a cell-cluster.

In order to find out whether the rise of $[Ca^{2+}]_i$ represented an influx of calcium from the medium into the cell, or the release of calcium from intracellular calcium stores, cells were treated with DCVC (0.5 mM) in a nominally calcium-free incubation buffer. This did not prevent the DCVC-induced rise of $[Ca^{2+}]_i$ (Fig. 4). When cells were incubated in a buffer containing 1.0 mM Ca^{2+} and 2.0 mM EGTA (EGTA-buffer), the same $[Ca^{2+}]_i$ was reached after a 90 min incubation-period with DCVC (0.5 mM) compared to cells treated with DCVC in a nominal calcium-free incubation buffer (data not shown). Incubation of cells either in a nominal calcium-free buffer or EGTA-buffer only, resulted in a slightly decreased $[Ca^{2+}]_i$ compared to that in normal, calcium-containing buffer.

The $\Delta\Psi$ decreased in control incubations to about 80% of the initial value after 90 min. DCVC (0.5 mM) caused a decrease of the $\Delta\Psi$, starting after approx. 50 min. After 90 min the $\Delta\Psi$ had dropped to 28% of the initial value (not shown).

During the incubation period no cell death was observed, neither in control nor in DCVC treated cells (as determined by the absence of intense staining of the nucleus with propidium iodide); cell death occurred after 110 min. This indicates that both a $[Ca^{2+}]_i$ increase and a decrease of $\Delta\Psi$ preceded DCVC-induced cell death.

To investigate whether chelation of intracellular calcium prevented the DCVC-induced decrease of $\Delta\Psi$, as already shown in the flow cytometry experiments, cells were incubated with DCVC (0.5 mM) together with the tetra-AM ester of the calcium chelator EDTA, EDTA/AM. EDTA/AM (5 μM) added 30 min prior to the addition of DCVC (0.5 mM) significantly delayed the decrease of $\Delta\Psi$ (not shown).

Relationship between DCVC-induced increase of $[Ca^{2+}]_i$ and decrease of $\Delta\Psi$

The above described effects on $[Ca^{2+}]_i$ and $\Delta\Psi$, suggest a relationship between the DCVC-induced increase of $[Ca^{2+}]_i$ and decrease of $\Delta\Psi$. Therefore this possible relationship was investigated in the individual cells of the population of cells incubated with either 0.25, 0.5 or 1.0 mM DCVC. This enabled us to test whether there was a correlation between the effects of DCVC on $[Ca^{2+}]_i$ and the $\Delta\Psi$ on a cell to cell basis. Four different types of DCVC effects were observed: an increase of $[Ca^{2+}]_i$ without an effect on $\Delta\Psi$, a decrease of $\Delta\Psi$ and an increase of $[Ca^{2+}]_i$ occurring simultaneously; an increase of $[Ca^{2+}]_i$ preceded by a decrease of $\Delta\Psi$ and a decrease of $\Delta\Psi$ without any increase of $[Ca^{2+}]_i$ of which representative time courses are shown in Fig. 5A–D, respectively. Table 2 summarizes the effects

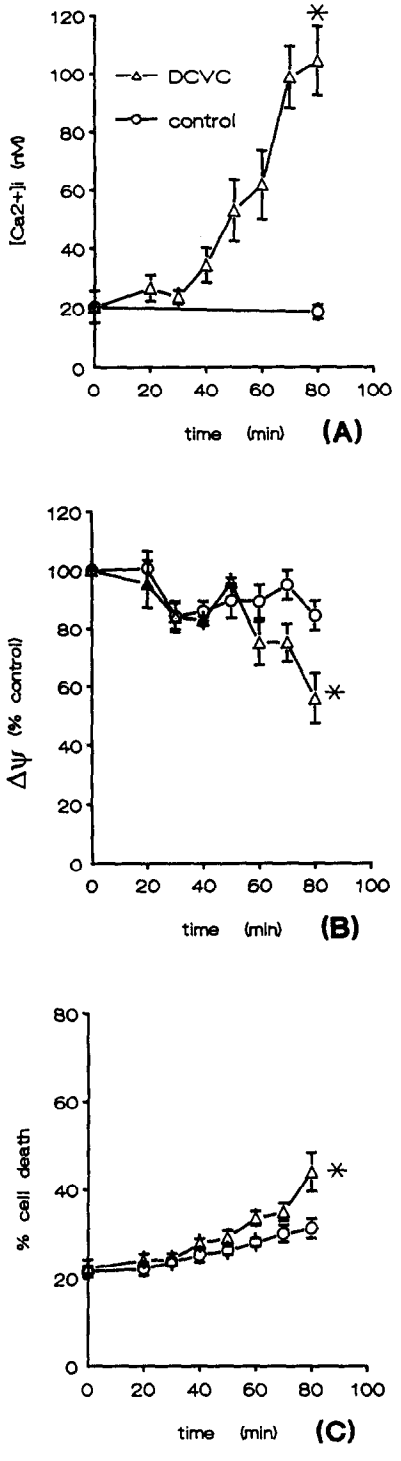

Fig. 3. Effect of DCVC on $[Ca^{2+}]_i$ in freshly isolated PTC in suspension. $[Ca^{2+}]_i$ (A) was determined fluorimetrically using Quin-2 as described in the Materials and Methods. $\Delta\Psi$ (B) and cell death (C) of the same population of cells as used for the measurement of $[Ca^{2+}]_i$, were determined by flow cytometry as described in Fig. 2. Shown are the means ± SEM (N = 5). Addition of ionomycine (6 μM) resulted in an increase in $[Ca^{2+}]_i$, within 1 min, from 18.5 ± 2.4 to 150 ± 20.6 nM (N = 5). Asterisk indicates significant difference from control (Student's *t*-test, $P < 0.05$).

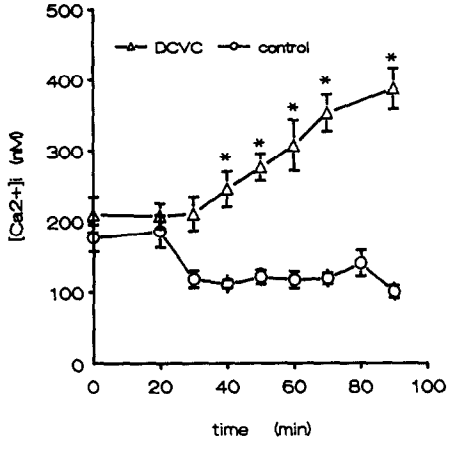

Fig. 4. Effect of omission of extracellular calcium on $[Ca^{2+}]_i$ in rat PTC. PTCs were treated as described in Fig. 2. After loading with FURA-2 and rhodamine-123, cells were washed three times with calcium-free HBSS/HEPES/BSA, prior to addition of buffer without calcium, containing 0.2 μM rhodamine-123 and 1 μM propidium iodide. DCVC (0.5 mM) was added 1 min after recording the first images at $t = 0$. Data represent mean ± SEM of 24–30 cells per data point, observed in at least five different experiments. Asterisk indicates significantly different from control (Student's *t*-test, $P < 0.05$).

observed for either 0.25, 0.5 or 1.0 mM DCVC. These data indicate that the DCVC-induced effects on $[Ca^{2+}]_i$ and the $\Delta\Psi$ can appear independently in individual cells. Further, the DCVC-induced decrease of the $\Delta\Psi$ seems to occur primarily at higher concentrations of DCVC, whereas the effect on $[Ca^{2+}]_i$ already takes place at lower concentrations.

DISCUSSION

The results show that DCVC has effects on both $[Ca^{2+}]_i$ and on $\Delta\Psi$ in PTC from rat kidney. The DCVC-induced change in intracellular calcium homeostasis depends on the release of calcium from intracellular stores since incubation in a nominally calcium-free buffer or an EGTA-buffer both resulted in an increase of $[Ca^{2+}]_i$. Whether calcium is released from the endoplasmatic reticulum, mitochondria or other intracellular calcium stores is unclear. High intracellular free calcium may by itself have adverse effects on mitochondria [5, 6]. There are two major pathways by which an increased $[Ca^{2+}]_i$ could potentiate DCVC-induced mitochondrial damage. Mitochondria can take up calcium by the uniport driven by the negative electrochemical gradient, thereby preventing a high cytosolic $[Ca^{2+}]$ (for reviews see: Refs 1, 2 and 29). Consequently, a continuous loading of the mitochondria with calcium may result in a release of calcium from the matrix by the Ca^{2+}/H^+ antiport [30, 31]. Released calcium may again be taken up by the mitochondrial uniport, leading to a continuous calcium cycling over the mitochondrial inner membrane, and an extensive energy drain. Alternatively, calcium-induced mitochondrial damage might be the result of activation of

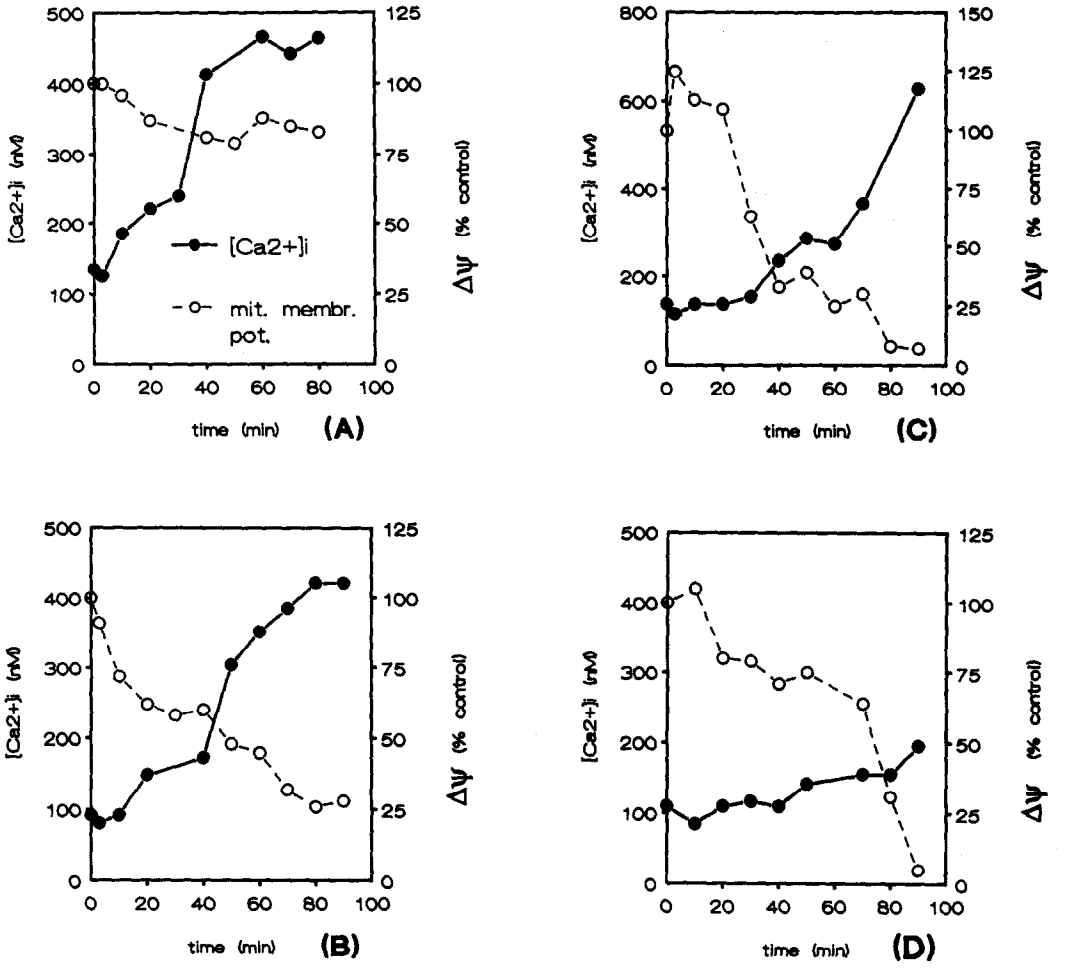

Fig. 5. The four different DCVC-induced effects on $[Ca^{2+}]_i$ and $\Delta\Psi$ observed by VIFM. Shown are representative examples of the different DCVC-induced effects on $[Ca^{2+}]_i$ and $\Delta\Psi$. An increase of $[Ca^{2+}]_i$ without an effect on $\Delta\Psi$ (A); an increase of $[Ca^{2+}]_i$ and a decrease of $\Delta\Psi$ occurring simultaneously (B); an increase of $[Ca^{2+}]_i$ preceded by a decrease of $\Delta\Psi$ (C) and a decrease of $\Delta\Psi$ without an effect on $[Ca^{2+}]_i$ (D). Representative time courses of the different effects observed in the population of cells incubated with 0.25, 0.5 and 1.0 mM DCVC are shown. Each figure represents the time course of an individual cell, where the cell (A) was incubated with 0.25 mM, (B) with 1.0 mM, (C) with 0.5 mM and (D) with 1.0 mM.

Table 2. Distribution of the effects of DCVC on $[Ca^{2+}]_i$ and the $\Delta\Psi$ observed in individual cells

Observed effect		% of total observed effects		
		Concentration DCVC (mM)		
$[Ca^{2+}]_i$ increase	$\Delta\Psi$ decrease	0.25	0.5	1.0
first	no effect	54	19	0
simultaneous		33	35	23
subsequent	first	0	23	46
no effect	first	13	23	31

The distribution of the four possible effects, in 24–30 observed cells is shown. Expressed is the % of the cells that gives a certain response.

mitochondrial phospholipases, such as phospholipase A_2, leading to the formation of free fatty acids [2, 32–34]. Accumulation of these free fatty acids will cause a permeability transition of the mitochondrial inner membrane [35–37]. These two mechanisms may further increase the mitochondrial damage already caused by DCVC metabolism directly.

The mitochondrial damage, involved in the DCVC-induced cytotoxicity to PTCs, is indeed related to changes in intracellular calcium, since the DCVC-induced decrease of the $\Delta\Psi$ was delayed by the intracellular calcium chelators EDTA/AM, EGTA/AM and Quin-2/AM. However, chelation of intracellular calcium did not prevent a DCVC-induced decrease of $\Delta\Psi$ and cell death. Thus, DCVC (after metabolism by β-lyase) seems to damage mitochondria additionally in a calcium independent way. Indeed it was shown in isolated rat kidney mitochondria in a calcium-free buffer, that DCVC inhibited state 3 and state 4 respiration (which was also dependent on β-lyase activity) [17].

The delay in the decrease of $\Delta\Psi$ and cell death by the chelation of calcium suggested a causal relationship: an increase of calcium was coupled to a decrease in $\Delta\Psi$. However, as discussed above, DCVC can also damage mitochondria directly without a need for calcium. Due to these two opposite mechanisms, three situations in individual cells (because of intracellular variability) are to be expected: a decrease of $\Delta\Psi$ and an increase of $[Ca^{2+}]_i$ occurring simultaneously; an increase of $[Ca^{2+}]_i$ preceded by a decrease of $\Delta\Psi$ and a decrease of $\Delta\Psi$ without any increase of $[Ca^{2+}]_i$. Indeed all three possible effects were observed, indicating that a decrease of $\Delta\Psi$ may occur independently or in combination with an increase of $[Ca^{2+}]_i$ in individual cells. In addition a fourth situation was observed: elevated $[Ca^{2+}]_i$ without a decrease of $\Delta\Psi$, indicating that also an increase of $[Ca^{2+}]_i$ is not strictly related to an effect on the $\Delta\Psi$. With 1.0 mM DCVC in 46% of the cells an initial decrease of $\Delta\Psi$ was soon followed by a drastic increase of $[Ca^{2+}]_i$ (see Fig. 5C), suggesting a release of calcium from the mitochondria due to either a non-specific pore opening or release via the mitochondrial uniporter [2]. However, at 0.25 mM DCVC an increase of calcium was observed in cells which did not show a decrease of the $\Delta\Psi$, indicating that calcium in those cells is presumably not released from the mitochondria, at least not by one of the above mentioned mechanisms. These data indicate that a rise of $[Ca^{2+}]_i$ may already take place at lower concentrations independent of mitochondrial damage whereas mitochondrial damage occurs primarily at higher concentrations.

In summary, the results show that DCVC induces an increase of $[Ca^{2+}]_i$ and a decrease of $\Delta\Psi$ in freshly isolated PTC and these effects may both appear independently in individual cells. Furthermore, the data indicate that the DCVC-induced increase of $[Ca^{2+}]_i$ potentiates the decrease of $\Delta\Psi$.

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