Mitochondrial K^+ as modulator of Ca^{2+} -dependent cytotoxicity in hepatocytes

Novel application of the K^+ -sensitive dye PBFI (K^+ -binding benzofuran isophthalate) to assess free mitochondrial K^+ concentrations

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In isolated rat hepatocytes a sustained high increase in intracellular free Ca^{2+} ($[Ca^{2+}]_i$), induced by extracellular ATP, is associated with mitochondrial dysfunction and cell death. The Ca^{2+} -induced effects are P_i -dependent and less severe when the intracellular K^+ content is low. In this study, the involvement of mitochondrial K^+ processing in Ca^{2+} -induced loss of mitochondrial membrane potential (MMP) and viability was investigated. The recently introduced K^+ -sensitive dye PBFI (K^+ -binding benzofuran isophthalate) has been used in combination with video-microscopy to assess intramitochondrial free K^+ concentration ($[K^+]_{mito}$) in rat liver mitochondria *in situ*. After

rapid permeabilization of the plasma membrane to remove cytosolic PBFI, the remaining PBFI was localized in mitochondria, and a 'resting' $[K^+]_{mito}$ of approx. 15 mM could be measured. Increased $[K^+]_{mito}$ levels were measured after induction of a prolonged increase in $[Ca^{2+}]_i$ by ATP. Much lower $[K^+]_{mito}$, more comparable with control levels, were observed when intracellular K^+ was depleted by omission of extracellular K^+ . In permeabilized cells the Ca^{2+} -induced, P_i -dependent, dissipation of the MMP was markedly delayed in the absence of K^+ . These observations suggest involvement of $[K^+]_{mito}$ as modulating agent in Ca^{2+} -induced cytotoxicity in hepatocytes.

INTRODUCTION

Changes in intracellular free Ca^{2+} ($[Ca^{2+}]_i$) are common in normal cellular functioning [1–5]. However, non-physiological sustained increases in $[Ca^{2+}]_i$ initiate processes which finally compromise cell viability [6–9]. Such pathological disturbance of the Ca^{2+} homeostasis is often associated with the development of cell injury induced by toxic compounds [10–17]. Mitochondria play an important role in Ca^{2+} homeostasis; they take up Ca^{2+} through a uniporter uptake mechanism, which is driven by the mitochondrial membrane potential (MMP) [18,19]. In isolated mitochondria excessive Ca^{2+} -uptake induces, under certain conditions, a permeability transition of the mitochondrial inner membrane, leading to dissipation of the MMP [18,20,21]. In intact hepatocytes we observed a similar accumulation of mitochondrial Ca^{2+} after induction of a sustained high increase in $[Ca^{2+}]_i$ by extracellular ATP [22]. This mitochondrial Ca^{2+} loading, with P_i as inducing agent, was associated with dissipation of the MMP and cell death.

Recently a role for intracellular K^+ in the Ca^{2+} -induced mitochondrial effects was suggested [9,23]. Depletion of K^+ in isolated mitochondria prevented the Ca^{2+} -dependent permeability transition induced by carboxyatractyloside [23]. Accordingly, we found in intact hepatocytes that depletion of cellular K^+ decreased Ca^{2+} -induced cytotoxicity [9]. Therefore we hypothesized that intramitochondrial K^+ levels ($[K^+]_{mito}$), which may be regulated by Ca^{2+} -activated channels in the inner membrane [24], are important for the development of Ca^{2+} -induced cytotoxicity. However, intramitochondrial free K^+ levels could not be determined so far, because no methodology existed for measurement of $[K^+]_{mito}$. Due to the recent introduction of a K^+ -sensitive fluorescence indicator, PBFI (K^+ -binding benzo-

furan isophthalate) [25,26], the assessment of $[K^+]_{mito}$ has now become feasible.

In the present study we report for the first time the determination of $[K^+]_{mito}$ in mitochondria using PBFI and video imaging. This technique was applied to assess the involvement of mitochondrial K^+ in Ca^{2+} -induced mitochondrial dysfunction after exposure of hepatocytes to ATP. We found that alteration in mitochondrial K^+ handling, reflected by high levels of $[K^+]_{mito}$, is essential in Ca^{2+} -induced cytotoxicity by extracellular ATP.

MATERIALS AND METHODS

Materials

Collagenase and ATP were obtained from Boehringer, Mannheim, Germany. BSA (type V), poly-D-lysine hydrobromide, rhodamine-123, digitonin, nigericin, valinomycin and propidium iodide were obtained from Sigma (St. Louis, MO, U.S.A.). PBFI/AM (acetoxymethyl ester) was purchased from Molecular Probes (Eugene, OR, U.S.A.).

Isolation and incubation of hepatocytes

Liver parenchymal cells were isolated by collagenase perfusion [27]. Cells were kept on ice until use. Cells were preincubated for 30 min at 37 °C before the start of the experiments, unless otherwise indicated. Cells were incubated at a density of 3×10^6 cells/ml in Hanks'/Hepes buffer (pH 7.4, 37 °C), composed of 120 mM NaCl, 4.2 mM $NaHCO_3$, 1.2 mM NaH_2PO_4 , 2.6 mM $CaCl_2$, 0.5 mM $MgSO_4$, 25 mM Hepes, supplemented with 10 mM glucose and 1.5% (w/v) BSA. Depending on the experimental conditions, the incubation medium contained no or 5 mM KCl.

Abbreviations used: $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; MMP, mitochondrial membrane potential; $[K^+]_{mito}$, intramitochondrial free K^+ concentration; $[K^+]_o$, extracellular K^+ concentration; PBFI, K^+ -binding benzofuran isophthalate; VIFM, video-intensified fluorescence microscopy; CCCP, carbonyl cyanide *p*-chlorophenylhydrazone.

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Flow-cytometric analysis of the MMP and cell viability

For determination of the MMP and cell viability in flow-cytometric studies, cell suspensions were loaded with 1.5 μ M rhodamine-123 and 2 μ M propidium iodide for 15 min at 37 °C. Cells were analysed for their fluorescence on a FACScan flow cytometer (Becton Dickinson, San Jose, CA, U.S.A.) as described previously [9]. Cellular uptake of rhodamine reflected cells exhibiting a MMP. Dead cells were identified by uptake of propidium iodide.

Video-Intensified fluorescence microscopy (VIFM) of $[K^+]_{mito}$

Cells were preincubated on a rotary shaker (200 cycles/min, 37 °C) for 45 min with or without extracellular K^+ . Then the cells were attached to glass coverslips coated with poly-D-lysine [9], which were mounted at the bottom of a thermostatically controlled incubation chamber (300 μ l volume, 37 °C) on the microscope. Images of individual hepatocytes were taken with a computer-controlled CCD series 200 camera system (Photometrics, Tucson, AZ, U.S.A.) and analysed with an Image system (Synoptics, Cambridge, U.K.). The VIFM system was described in detail previously [9,22].

A recently developed procedure allows mitochondrial Ca^{2+} concentration to be studied after loading of the mitochondrial compartment with a fluorescent probe [22,28]. We have adapted this methodology to assess $[K^+]_{mito}$. A coated glass cover-slip (diameter 1 cm), mounted in the incubation chamber, was covered with 300 μ l of cell suspension, to which 20 μ M PBFI/AM was added and incubated for 60 min at 37 °C. After hydrolysis of the ester, the K^+ -sensitive free acid PBFI remains trapped within the cytosol as well as the mitochondria. The cells were washed with Hanks'/Hepes buffer to remove extracellular dye. Approx. 1.5×10^4 cells were attached to the coverslip. Then the cells were washed with a sucrose buffer containing 250 mM sucrose, 25 mM Tris, 3 mM EGTA, 5 mM $MgCl_2$, 5 mM succinate and 5 mM glutamate (37 °C, pH 7.3). As with other probes for ion determination, the cells showed a uniform diffuse fluorescence. In order to remove the cytosolic PBFI and to control the extramitochondrial conditions, the plasma membrane was permeabilized by addition of digitonin (final concn. 150 μ M). After 25 s permeabilization was complete, which was indicated by the appearance of non-fluorescent nuclei surrounded by a ring of granular fluorescence (Figure 1b). In the next 5 s the microscope was focused on the nuclei, and digital imaging of the remaining fluorescence was performed at 340 and 380 nm excitation and 495 nm emission. The ratio of 340/380 nm excitation images was used to calculate $[K^+]_{mito}$ [25,26]. For each experiment five to ten cells were analysed.

To validate that fluorescence after permeabilization originated from PBFI localized in mitochondria, cells were loaded with PBFI/AM as described above, and co-loaded for 15 min with 350 nM rhodamine-123; the latter dye accumulates in mitochondria. After permeabilization with digitonin, fluorescence of both PBFI (340/380 nm excitation) and rhodamine (450–490 nm excitation) displayed a granular pattern in the cells (Figures 1a and 1b). Under the present conditions, the contribution of one probe to the other's emission was negligible. The distribution of both probes was virtually identical, indicating a predominant mitochondrial localization of PBFI.

To check further whether some PBFI located outside the mitochondria might affect the finally measured value of $[K^+]_{mito}$, the spots which showed rhodamine fluorescence were mapped on to the ratio image of PBFI. Then the mean PBFI ratio value within those spots exclusively was measured; it proved to be

identical with the mean ratio value of the total PBFI-fluorescent area. This was observed in all the experimental conditions described (in control as well as treated cells).

VIFM of the MMP

To study the MMP, cells were loaded with 1.5 μ M rhodamine for 15 min and attached to the coverslips simultaneously. Then the cells were washed three times with Ca^{2+} -free Hanks'/Hepes buffer and incubated in a K^+ -containing or K^+ -free sucrose buffer composed respectively of 100 mM KCl/50 mM sucrose or 250 mM sucrose. To both buffers 25 mM Tris, 5 mM $MgCl_2$, 5 mM succinate and 5 mM glutamate were added (37 °C, pH 7.3). The cells were then permeabilized as described above. Fluorescence was measured by using a 450–490 excitation filter, a 510 nm dichroic mirror and a 520 nm long-pass filter.

In permeabilized cells, fluorescence of mitochondria, loaded with rhodamine, but uncoupled with carbonyl cyanide *p*-chlorophenylhydrazone (CCCP) (final concn. 5 μ M), was taken as a background value and subtracted from all images.

RESULTS

Effect of omission of extracellular K^+ on Ca^{2+} -induced dissipation of MMP and cell death after exposure to ATP

Flow-cytometric analysis was used to study the effect of high $[Ca^{2+}]_i$ on MMP and viability. In agreement with previous results [9], induction of high $[Ca^{2+}]_i$ by ATP for 2 h induced extensive loss of viability, and loss of MMP in the remaining viable cells (the propidium iodide-negative cells). Depletion of intracellular K^+ by incubation in K^+ -free medium decreased the loss of viability and MMP (Table 1). Since changes in $[K^+]_{mito}$ might be involved in these Ca^{2+} -induced effects, we decided to study changes in mitochondrial K^+ handling. In order to do so, we developed a new methodology using the K^+ -sensitive dye PBFI.

Fluorescence measurement of $[K^+]_{mito}$

To assess $[K^+]_{mito}$ with VIFM, a procedure was adapted that was previously validated to determine mitochondrial Ca^{2+} levels with VIFM [22,28]. Cells attached to coverslips were loaded with PBFI/AM. The extramitochondrial PBFI was removed by permeabilization of the plasma membrane with digitonin. In this process the intracellular medium equilibrated with a K^+ -free

Table 1 Effect of omission of extracellular K^+ on ATP-induced cell death and dissipation of the MMP

Isolated hepatocytes were incubated in Hanks'/Hepes with or without K^+ , and exposed for 2 h to 0.4 mM ATP. Samples (0.5 ml) loaded with 1.5 μ M rhodamine-123 and 2 μ M propidium iodide were analysed for their fluorescence on the flow cytometer. The viability is expressed as the percentage of cells which excluded propidium iodide. The MMP of the viable cells (calculated by averaging their rhodamine fluorescence) is expressed as a percentage of the control at $t = 0$. Data shown are mean values $\pm$ S.E.M. of three separate isolations: * $P < 0.05$ versus control without ATP; † $P < 0.05$ versus corresponding value in the presence of K^+ .

Treatment (2 h)	Viability (%)	MMP (% of control at $t = 0$)
+ K^+ , control	89 $\pm$ 1	90 $\pm$ 4
+ K^+ , ATP	34 $\pm$ 4*	64 $\pm$ 6*
No K^+ , control	89 $\pm$ 1	99 $\pm$ 6
No K^+ , ATP	61 $\pm$ 5*†	87 $\pm$ 8

Figure 1 Fluorescence images of permeabilized hepatocytes loaded with PBFI/AM and rhodamine-123 simultaneously before permeabilization

Hepatocytes attached to glass coverslips were incubated with $20 \mu M$ PBFI/AM for 60 min and co-loaded with $350 nM$ rhodamine-123 for 15 min. To remove cytosolic components, the plasma membranes were permeabilized by addition of digitonin in sucrose buffer before images were taken. (a) Digital fluorescence image of rhodamine-123 (excitation $450\text{--}490 nm$), an indicator of MMP. (b) Digital fluorescence image of PBFI (excitation $340 nm$) in the same cells.

sucrose buffer. The remaining PBFI was localized in mitochondria (see the Materials and methods section), as was validated after co-localization with rhodamine-123 (Figures 1a and 1b). The mitochondrial PBFI was calibrated by clamping $[K^+]_{mito}$ to known values of $[K^+]_o$ (extracellular $[K^+]$) by using the standard sucrose buffer supplemented with the mitochondrial uncoupler CCCP ($1.5 \mu M$) and the ionophores valinomycin ($5 \mu M$) and nigericin ($5 \mu M$). Under these conditions $[K^+]_{mito}$ equilibrates with $[K^+]_o$ [25,29,30] and digital fluorescence imaging was performed while $[K^+]_o$ was varied. By plotting the $340/380 nm$ excitation ratio against $[K^+]_o$, a standard curve was generated (Figure 2).

The data fitted to the equation reported by Kasner and Ganz [25]:

$$I_{340}/I_{380} = (I_{max} \cdot [K^+]_{mito}) / ([K^+]_{mito} + K_m) + I_0$$

in which I_{340}/I_{380} is the measured fluorescence ratio, I_0 the ratio

Figure 2 Calibration curve for PBFI

Cells attached to glass coverslips were loaded with PBFI-AM. After permeabilization of the plasma membrane, the cells were incubated in a sucrose buffer supplemented with CCCP, valinomycin and nigericin (see the Materials and methods section) to clamp $[K^+]_{mito}$ to known values of $[K^+]_o$. Images were taken to measure fluorescence; the ratio ($340/380 nm$) is plotted against $[K^+]_o$. Per experiment, 5–10 cells were examined. Samples of four separate isolations were measured for each value of $[K^+]_o$; error bars indicate S.E.M.

in the absence of $[K^+]_{mito}$, the sum of I_{max} and I_0 is the maximal ratio (as $[K^+]_{mito}$ approaches infinity) and K_m reflects the affinity of the dye for K^+ . By using the non-linear least-squares regression program Siphar (Simed SA, Creteil, France), the fitting determined an I_0 of 0.85 and an I_{max} of 1.87, with a K_m of $39 mM$. This calibration curve was used to calculate $[K^+]_{mito}$ from the measured fluorescence ratios. Construction and fitting of a standard curve generated with the free acid PBFI in the sucrose buffer described ($37^\circ C$, $pH 7.3$) revealed a K_m of $6 mM$ in the absence of Na^+ and $8 mM$ in the presence of $5 mM Na^+$.

Because PBFI also exhibits some affinity for Na^+ [26], we measured intramitochondrial free $[Na^+]$ with the Na^+ -selective dye SBFI (Na^+ -binding benzofuran isophthalate) (results not shown) by using the same protocol as described for the $[K^+]_{mito}$ measurements. After permeabilization of control or ATP-treated cells, intramitochondrial free $[Na^+]$ appeared to be very low ($< 5 mM$) compared with $[K^+]_{mito}$ and therefore not interfering with our K^+ measurements.

Ca^{2+} -induced effects on $[K^+]_{mito}$ after exposure to ATP

Cells, attached to coverslips and loaded with PBFI/AM, were permeabilized after 60 min with digitonin (see the Materials and methods section) and $[K^+]_{mito}$ was measured. In this process, a K^+ -free sucrose buffer was used to prevent uptake of K^+ during and after permeabilization. Mitochondria of control cells exhibited a $[K^+]_{mito}$ of approx. $15 mM$ (Table 2).

To study the effect of a sustained high increase in $[Ca^{2+}]_i$ on mitochondrial K^+ handling, $0.4 mM$ ATP was added to the cells 5 min before they were attached to the coverslips and loaded with PBFI/AM. Then 60 min later the cells were permeabilized in K^+ -free sucrose buffer and $[K^+]_{mito}$ was determined. Cells exposed to ATP exhibited a strikingly high level of $[K^+]_{mito}$. However, $[K^+]_{mito}$ was clearly lower when the cells were exposed to ATP in the absence of extracellular K^+ (Table 2). The $[K^+]_{mito}$ of control or treated cells after permeabilization remained constant for at least 2.5 min (results not shown).

Table 2 Effect on $[K^+]_{mito}$ after exposure to ATP

Cells, incubated with 0.4 mM ATP in the absence or presence of extracellular K^+ , were attached to the coverslips and loaded with PBFI-AM. After exposure for 60 min, cells were permeabilized with digitonin and fluorescence images were taken. The calibration curve (Figure 2) was used to convert the measured fluorescence ratios into values of $[K^+]_{mito}$. Data shown are mean values $\pm$ S.E.M. of three to four separate isolations: * $P < 0.01$ compared with control and value in the absence of K^+ .

Treatment (1 h)	$[K^+]_{mito}$ (mM)
Control, no K^+	17.5 ± 1.8
Control, + K^+	14.2 ± 1.6
ATP, no K^+	25.5 ± 1.7
ATP, + K^+	$44.8 \pm 4.5^*$

Figure 3 Effect of K^+ on the Ca^{2+} -induced loss of MMP

Attached hepatocytes were loaded with rhodamine-123. After permeabilization ($t = 0$) of the plasma membrane, the cells were incubated in a K^+ -free or a K^+ -containing sucrose buffer (see the Materials and methods section). After 30 s, 50 μ M $CaCl_2$ was added. At various time points fluorescence images were taken to study MMP. Per experiment, 8–15 cells were examined. Media: ●, 250 mM sucrose; ○, 100 mM KCl/50 mM sucrose; ▲, 100 mM KCl/50 mM sucrose/3 mM EGTA. Data are means $\pm$ S.E.M. of four separate isolations: * $P < 0.001$ compared with value in the presence of EGTA or in the absence of K^+ ; † $P < 0.05$ compared with value in the presence of EGTA.

Effect of $[K^+]_{mito}$ on Ca^{2+} -induced dissipation of MMP

The MMP of individual cells was studied by VIFM. Cells attached to coated glass coverslips were loaded with rhodamine-123. After permeabilization of the plasma membrane with digitonin, the cells were incubated in the sucrose buffer, as described in the Materials and methods section, composed of 100 mM KCl and 50 mM sucrose. Fluorescence images were collected to study the MMP. After the first image ($t = 0$), 50 μ M $CaCl_2$ was added. Mitochondrial fluorescence decreased rapidly after approx. 15 min, reflecting dissipation of the MMP (Figure 3). However, when mitochondria were exposed to Ca^{2+} in a K^+ -free buffer, to prevent mitochondrial K^+ influx, the dissipation of the MMP was strongly delayed (Figure 3). To assess the involvement of Ca^{2+} , EGTA was added to the medium. In this

Figure 4 Effect of P_i on the Ca^{2+} -induced loss of MMP

Permeabilized cells loaded with rhodamine-123 were incubated in a sucrose buffer containing 100 mM K^+ . At $t = 0.5$ min, 50 μ M $CaCl_2$ was added (see Figure 3). Media: ●, 20 mM P_i ; ○, 4 mM P_i ; ▲, 20 mM P_i /3 mM EGTA. Data are means $\pm$ S.E.M. of at least three separate isolations: * $P < 0.001$ compared with value in the presence of 4 mM P_i ; † $P < 0.02$, and ‡ $P < 0.001$, compared with value in the presence of EGTA.

case the mitochondria maintained a MMP, which indicated that the loss of MMP described above was dependent on Ca^{2+} (Figures 3 and 4). Recently we reported that the Ca^{2+} -induced effects after exposure to ATP in intact hepatocytes was markedly exacerbated by P_i [22]. A Ca^{2+} -dependent collapse of the MMP, induced by P_i , was suggested as the underlying mechanism. Therefore the role of P_i as inducer was evaluated by increasing the P_i concentration in the incubation medium. Under these conditions the collapse of the MMP occurred markedly earlier (Figure 4).

DISCUSSION

Recently we have shown that sustained high $[Ca^{2+}]_i$ levels in hepatocytes caused by exposure to extracellular ATP are associated with loss of MMP and, finally, cell death [9]. The cytotoxic effect of high $[Ca^{2+}]_i$ in hepatocytes is decreased by lowering intracellular $[K^+]$ [9]. In the present paper we studied the involvement of $[K^+]_{mito}$ as modulator of the P_i -dependent mitochondrial dysfunction induced by high $[Ca^{2+}]_i$ after exposure to ATP.

PBFI as probe for $[K^+]_{mito}$

To examine the involvement of $[K^+]_{mito}$, we applied the recently introduced K^+ indicator PBFI in digital fluorescence imaging. Since, to our knowledge, this is the first reported attempt to observe and quantify $[K^+]_{mito}$, we performed a number of control experiments. After permeabilization of PBFI-loaded hepatocytes, fluorescence due to mitochondrial accumulation of the dye could be measured. The mitochondrial localization of the fluorescence was confirmed by VIFM methodology. We found that mitochondrial PBFI fluorescence is altered upon changes in K^+ within the range 0–75 mM K^+ , with a K_m of 39 mM. This K_m value was lower than that reported by Kasner and Ganz [25] in intact cells, which probably is due to the fact that in our experiments Na^+ is continuously low, in contrast with their conditions, in which the

sum of the Na^+ and K^+ concentrations was kept constant [25]. Our 'mitochondrial K_m ' is different from the K_m found with PBFI in buffer. However, this is not uncommon: many probes have an altered affinity for their cation when sequestered *in situ*, because of interference with intracellular constituents [29,31].

Role of $[K^+]_{mito}$ in Ca^{2+} -induced cytotoxicity

Until now, $[K^+]_{mito}$ could not be measured. Previous reports on total mitochondrial K^+ contents [32,33] and mitochondrial volume [24] suggested that the mitochondrial K^+ concentration was low compared with that in the cytosol: approx. 40–50 mM. Another report [34] showed a 3.4-fold lower amount of K^+ (per g of nitrogen) in the mitochondria as compared with the cytosol, resulting in a calculated $[K^+]_{mito}$ of 25–30 mM. It is not unlikely that a (substantial) fraction of this K^+ is bound, as is the case with other cations such as Na^+ , Mg^{2+} and Ca^{2+} [29,35,36]. Therefore, it is not surprising that the $[K^+]_{mito}$ is as low as we observed. After exposure to a long-lasting, ATP-induced, increase in $[Ca^{2+}]_i$, the mitochondria exhibited a much increased level of free K^+ . This implied that either mitochondrial K^+ influx was enhanced or transport of K^+ out of the mitochondria was impaired, as we predicted in our hypothesis. Under conditions of low intracellular K^+ (induced by omission of extracellular K^+), the high $[Ca^{2+}]_i$ resulted in a much lower $[K^+]_{mito}$.

Role of $[K^+]_{mito}$ in Ca^{2+} -induced loss of MMP

In permeabilized cells, dissipation of the MMP induced by high concentrations of Ca^{2+} was directly studied. Omission of K^+ provided a significant protection against membrane depolarization, suggesting a role for K^+ as modulator. Ca^{2+} chelation prevented the collapse of the MMP; in combination with the strongly enhancing effect of a high P_i concentration (as occurs intracellularly after exposure to ATP in intact hepatocytes [22]), this supports the proposed mechanism underlying Ca^{2+} -induced cytotoxicity by ATP. Omission of P_i did not completely protect against a collapse of the MMP (results not shown), indicating that P_i , in spite of its potency to promote, is not absolutely required. However it cannot be excluded that very small amounts of P_i , still present in the incubation medium, were enough to induce dissipation of the MMP.

The present data show that altered mitochondrial K^+ handling is associated with Ca^{2+} -induced loss of MMP and viability. In addition, non-toxic concentrations of mitochondrial K^+/H^+ -antiporter inhibitors strongly enhanced the Ca^{2+} -induced cytotoxicity by ATP (J. P. Zoetewij, unpublished work). Maintenance of the MMP is thought to be crucial for cellular integrity [9,37]. Several processes, such as mitochondrial NADPH depletion, mitochondrial Ca^{2+} release and/or rapid ATP hydrolysis, are thought to be critical consequences of mitochondrial de-energization. The mechanism of interaction between Ca^{2+} and K^+ , leading to mitochondrial dysfunction, remains to be established. An uncontrolled influx of K^+ into mitochondria, due to Ca^{2+} activation of K^+ channels [9,24] and/or non-specific membrane leakiness to small solutes, might be involved in these cytotoxic Ca^{2+} effects. In addition, a lower binding capacity of the mitochondrial matrix for Ca^{2+} in the presence of high matrix free K^+ may be involved [23].

In conclusion, we found that a decrease in MMP, induced by high $[Ca^{2+}]_i$ levels, correlated with a higher mitochondrial K^+ retention, suggesting that the decrease of MMP is caused by a high mitochondrial K^+ concentration.

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