Calcium-induced Cytotoxicity in Hepatocytes after Exposure to Extracellular ATP Is Dependent on Inorganic Phosphate

EFFECTS ON MITOCHONDRIAL CALCIUM*

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In isolated mitochondria extensive uptake of Ca$^{2+}$ in the presence of an "inducing agent," e.g. inorganic phosphate (P$_i$), causes permeabilization of the mitochondrial inner membrane and a collapse of the mitochondrial membrane potential. In this study we tested whether the effect of phosphate occurs in intact hepatocytes. Rat hepatocytes were incubated with ATP to induce a sustained increase in intracellular Ca$^{2+}$ ([Ca$^{2+}$]$_i$), dissipation of the mitochondrial membrane potential, and cell death (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). Omission of P$_i$ from the incubation medium delayed the loss of viability. The nonhydrolyzable ATP analog adenosine 5'-O-(3-thiotriphosphate) (ATP$_{y}$S) had similar effects on [Ca$^{2+}$]$_i$ and viability, but now omission of extracellular P$_i$ completely protected against cytotoxicity. Exposure to ATP or ATP$_{y}$S induced a large cellular uptake of P$_i$, completely protected against cytotoxicity. A prolonged increase in [Ca$^{2+}$]$_i$ is often associated with the development of cellular toxicity as occurs after exposure to toxic compounds or anoxia (15–23). The mitochondrial calcium uptake and extrusion system may be challenged by the increased Ca$^{2+}$ and eventually become compromised, leading to mitochondrial dysfunction and cell death. In a recent paper we showed that cell death induced by high [Ca$^{2+}$]$_i$ in hepatocytes, after exposure to extracellular ATP, is preceded by dissipation of the mitochondrial membrane potential (24). Influx of potassium, the major intracellular cation, into mitochondria, driven by the mitochondrial membrane potential, occurs when the mitochondrial inner membrane becomes more permeable (25, 26). A lowered [K$^+$]$_i$, reduced dissipation of the mitochondrial membrane potential and cytotoxicity induced by high calcium (24).

Whereas studies in isolated mitochondria have shown that phosphate stimulates mitochondrial calcium uptake and permeabilization of the mitochondrial inner membrane (8), it is unknown if these findings with isolated mitochondria are also applicable to intact cells. Therefore we have evaluated the possible role of phosphate as inducing agent in calcium-dependent cellular dysfunction after exposure to ATP.

EXPERIMENTAL PROCEDURES

Materials—Collagenase, ATP, and ATP$_{y}$S were obtained from Boehringer Mannheim, Germany. Bovine serum albumin (type V), poly-d-lysine hydrobromide, rhodamine 123, propidium iodide, digi-tonin, Fura-2/AM, and Firefly Lantern extract were obtained from Sigma. $^{32}$P$_i$ was purchased from Du Pont de Nemours, Bad Homburg, Germany.

Isolation and Incubation of Hepatocytes—Liver parenchymal cells were isolated by collagenase perfusion as reported previously (27). Cells were incubated at a density of 3 x 10$^6$ cells/mL in Hanks's solution/HEPES buffer (pH 7.4, 37°C) composed of 120 mM NaCl, 5 mM KCl, 4.5 mM NaHCO$_3$, 1.5 mM Na$_2$HPO$_4$, 2.6 mM CaCl$_2$, 0.5 mM

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The abbreviations used are: [Ca$^{2+}$]$_i$, intracellular free Ca$^{2+}$ concentration; [Ca$^{2+}$]$_{mito}$, mitochondrial free calcium concentration; [ATP]$_i$, intracellular ATP concentration; VIMF, video intensified fluorescence microscopy; CCCP, carbonyl cyanide m-chlorophenylhydrazone; ATP$_{y}$S, adenosine 5'-O-(3-thiotriphosphate).
MgSO₄, 25 mM HEPES, supplemented with 1% (w/v) glucose and 1.5% (w/v) bovine serum albumin.

**Flow Cytometric Analysis of Cell Viability**—For determination of cell viability in flow cytometric studies 7 μl of a 25 μM aqueous propidium iodide solution were added to 100 μl of the cell suspensions, and the cells were directly analyzed for their fluorescence on a FACSscan flow cytometer (Becton Dickinson, San Jose, CA). A detailed description of the FACSscan system has been given elsewhere (24). The number of cells which exhibited fluorescence due to propidium iodide uptake reflected the number of cells which had lost viability.

**Video Intensified Fluorescence Microscopy (VIFM) of [Ca²⁺]i, and Mitochondrial Free Ca²⁺ ([Ca²⁺]mito)**—For fluorescence measurements in single hepatocytes, the cells were attached to glass coverslips coated with poly-D-lysine. The attachment procedure as well as a detailed description of the VIFM system have been described recently (24). Briefly, the VIFM system consists of a Zeiss IM-35 inverted microscope (Oberkochen, Germany), Nikon Fluor 40× oil objective, and a CCD series 200 camera system (Photometrics, Tucson, AZ) controlled by a Compaq 386/20 computer (Compaq Computer Corporation, Houston, TX). Image analysis was performed with a Imagine system (Sympatic, Cambridge, U. K.).

For measurement of [Ca²⁺], and [Ca²⁺]mito (24, 28), hepatocytes were loaded with 20 μM Fura-2/AM for 60 min, after which the cells were washed with Hanks’ solution/HEPES buffer, mounted in an incubation chamber, and transferred to the microscope. Digitized fluorescence images were performed at 340- and 380-nm excitation and 505-nm emission. The ratio image of 340/380-nm excitation images was used to calculate [Ca²⁺] (29).

For determination of [Ca²⁺]mito (28) the Hanks’ solution/HEPES buffer was replaced by a sucrose buffer containing 210 mM sucrose, 3 mM HEPES, 10 mM K₃HPO₄, 5 mM glutamate, 5 mM succinate, and 5 mM MgCl₂.

The plasma membrane was subsequently permeabilized by addition of digitonin (final concentration 150 μM). Ir duplicate samples treated with digitonin, permeabilization of the plasma membrane was confirmed by cellular uptake of trypan blue (30). A few seconds after permeabilization, 340/380-nm images of the remaining fluorescence were taken, and ratio imaging was performed for estimation of [Ca²⁺]mito. To minimize intercellular variations in the determination of [Ca²⁺]mito due to thickness of the cells, the ratios were always determined of mitochondria present in the focal plane of the nuclei.

To confirm that the remaining fluorescence was due to P-2 localized in the mitochondria, the mitochondria were uncoupled with CCCP (final concentration 5 μM), which causes release of mitochondrial calcium. Indeed, after addition of CCCP the measured [Ca²⁺]mito dropped to nearly zero, which indicated its mitochondrial localization.

To check that the mitochondria were still intact after permeabilization, cells were loaded with rhodamine 123, which is a fluorescent indicator for the mitochondrial membrane potential (28, 31), before permeabilization. After treatment with digitonin-containing sucrose buffer, fluorescence of rhodamine was still observed. However, after treatment with digitonin and CCCP, fluorescence was lost due to desensitization of the mitochondrial membrane.

**Determination of Intracellular P₃ and ATP—**A 7-ml sample of the cell suspensions was taken, and the viable cells were separated from the medium and dead cells using a rapid centrifugation technique (32–34). In a tube 2 ml of dibutyl phthalate were layered onto 0.5 ml of 10% (v/v) aqueous HClO₄. The cell samples were layered on top of the dibutyl phthalate after which the tubes were centrifuged for 15 s at high speed (2500 × g). The viable cells were spun down into the HClO₄ fraction, leading to precipitation of cellular protein. Nonviable cells as well as the incubation medium itself remained on top of the dibutyl phthalate layer.

In a control experiment, cells were completely lysed with 150 μM digoxin (which caused permeabilization of the plasma membrane) and fractionated on the dibutyl phthalate/HClO₄ as described above. Lactate dehydrogenase was recovered in the upper fraction, and no precipitated protein was found in the HClO₄ fraction indicating that only viable cells move into the HClO₄.

Phosphate was assayed in the HClO₄ fractions using the molybdate assay (35), which was also used to measure phosphate in the medium. ATP was assayed by an optimized luciferin/luciferase method (36, 37).

**32P₀, uptake—**To determine uptake of [³²P]phosphate, cells were incubated in Hanks’ solution/HEPES buffer containing 1 μCi/ml [³²P]phosphate (specific activity 8500–9100 Ci/mmol). At various time points cell samples were fractionated by the above described rapid centrifugation technique. A sample of the supernatant of the HClO₄ fraction was mixed with Emulsifier Safe (Packard), and the radioactivity was measured by liquid scintillation counting. In these experiments [³²P]phosphate uptake is expressed relative to the uptake determined after 1-min incubation of the cells with [³²P]phosphate at 4° C.

**RESULTS**

**Dependence of ATP-induced Cytotoxicity on Extracellular Phosphate—**The dependence of cell death induced by extracellular ATP on extracellular P₃ was studied by incubating the cells in the absence or presence of extracellular P₃. The hepatocytes were analyzed by flow cytometry after addition of 2 μM propidium iodide to the medium. After 3 h of exposure to ATP in the presence of 1.2 mM P₃, uptake of propidium iodide (indicating cell death) in a large number of cells was observed (Fig. 1). Release of lactate dehydrogenase from the cells into the incubation medium occurred parallel with uptake of propidium iodide (data not shown). When phosphate was omitted from the incubation medium a significant delay in cell death was measured. Increasing the concentrations of phosphate in the medium above 1.2 mM only slightly increased loss of viability (data not shown).

Extracellular ATP is hydrolyzed at the plasma membrane to generate inorganic phosphate (38) and therefore addition of ATP to a P₃ deficient medium will result in replenishment of the P₃. Indeed, after addition of 0.4 mM ATP to the cells 0.9 mM phosphate was generated in the incubation medium linearly in 60 min (results not shown). This formation of P₃ made it difficult to study the importance of extracellular P₃.

To study cytotoxic effects induced by high levels of [Ca²⁺], without generation of extracellular P₃, the nonhydrolyzable ATP analog ATP₃S was used (39–41). Exposure to 1 mM ATP₃S caused an immediate, sustained increase in [Ca²⁺], equivalent to the increase induced by 0.4 mM ATP (measured with Quin-2 fluorimetry (20, 42), results not shown). The presence or absence of extracellular P₃ did not influence the induced rises in [Ca²⁺], by either ATP or ATP₃S. ATP₃S appeared to have similar cytotoxic effects as that of ATP: the loss of viability observed after 3 h of exposure to 1 mM ATP₃S was comparable to the extent of cell death observed after exposure to ATP. However, omission of phosphate from the

![FIG. 1. Effect of omission of extracellular P₃ on cell death induced by ATP.](image-url)
phosphate has been shown to be directly involved in mitochondrial 
crease in intracellular Pi, [ATP], was also determined. As previously 
described cellular Pi by
crease (Fig. 4).
Pi was expected to affect the intracellular Pi content. Indeed,
uptake of phosphate by the cells was involved in ATP-induced 
cytotoxicity. To test this hypothesis the cells were exposed to 
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The Pi dependence of the effects could imply that a net 
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**Effects on Intracellular Phosphate—**Uptake of extracellular Pi was expected to affect the intracellular Pi content. Indeed, after addition of 0.4 mM ATP extracellular Pi markedly increased (Fig. 4). Omission of extracellular Pi; reduced intracellular Pi; by 30% in the control and strongly delayed the increase in intracellular Pi, after exposure to ATP. To exclude intracellular ATP depletion as a cause of the measured increase in intracellular Pi, [ATP], was also determined. As previously described (20), [ATP], greatly increased after exposure to extracellular ATP (Fig. 5). Omission of extracellular Pi, delayed the increase in [ATP].

**Determination of [Ca²⁺]_{mito}—**In isolated mitochondria phosphate has been shown to be directly involved in mitochondrial 

calcium handling (8). With use of a VIFM technique changes in mitochondrial calcium in individual cells were measured (28). Ten minutes after addition of ATP the cells were attached to glass coverslips and loaded with Fura-2/AM. [Ca²⁺]_{mito} was determined after 70 min of exposure to ATP just before the onset of cell death (see "Experimental Procedures"). Later measurements, when extensive loss of viability had occurred, could not be done because this interfered with the attachment of the cells to the coverslips. Most cells exhibited a highly increased level of [Ca²⁺], and in addition a more 2-fold increase in [Ca²⁺]_{mito} (Fig. 6) upon addition of ATP. Omission of extracellular Pi, prior to exposure to ATP, significantly reduced the number of cells with an increased [Ca²⁺]_{mito} but had no effect on the measured [Ca²⁺], levels.

**DISCUSSION**

Recently we reported that extracellular ATP induced a prolonged high increase of [Ca²⁺], in isolated hepatocytes, which was associated with dissipation of the mitochondrial 

**Fig. 2.** Effect of omission of extracellular Pi on cell death induced by ATPyS. Control (●) and 1 mM ATPyS (■) in the presence of extracellular Pi; control (○) and 1 mM ATPyS (□) in the absence of extracellular Pi. Data are means ± S.E. of four separate isolations. *, p < 0.05; †, p < 0.005, compared with exposure to ATPyS in the absence of extracellular Pi.

**Fig. 3.** Cellular uptake of ³²P after exposure to ATP or ATPyS. Cells were incubated in Hanks' solution/HEPES buffer containing 1 μM/ml ³²P. At various time points cellular radioactivity was determined. In viable cells uptake of radioactivity after 1 min at 4°C was taken as 100%. Control (●), 0.4 mM ATP (△), and 1 mM ATPyS (Δ). Data are means ± S.E. of four separate isolations. *, p < 0.02; †, p < 0.01, compared with control.

**Fig. 4.** Effect of ATP on intracellular Pi, in viable cells. At various time points intracellular Pi, was determined. Control (●) and 0.4 mM ATP (■) in the presence of extracellular Pi; control (○) and 0.4 mM ATP (□) in the absence of extracellular Pi. Results are given as percentage of the control at t = 0. Data are means ± S.E. of four separate isolations. *, p < 0.01; †, p < 0.001, compared with control.

**Fig. 5.** Effect of ATP on [ATP], in viable cells. At various time points intracellular [ATP], was determined. Control (●) and 0.4 mM ATP (■) in the presence of extracellular Pi; control (○) and 0.4 mM ATP (□) in the absence of extracellular Pi. Results are given as percentage of the control at t = 0. At the beginning of the experiment control cells contained 11.7 ± 2.3 nmol of ATP/mg protein. Data are means ± S.E. of four separate isolations. *, p < 0.01; †, p < 0.03, compared with control.
membrane potential and, finally, cell death (20, 24). These deleterious effects of high [Ca\textsuperscript{2+}] were dependent on [K\textsuperscript{+}], supporting a mechanism in which permeabilization of the mitochondrial inner membrane causes leakage of small solutes and mitochondrial dysfunction (24).

Studies with isolated mitochondria showed that high calcium levels in the mitochondrial matrix are required for permeabilization (8). In the presence of a so-called inducing agent permeabilization is rapidly initiated. The purpose of this study was to evaluate in intact hepatocytes the role of phosphate as a possible inducing agent in the calcium-dependent cytotoxicity of extracellular ATP.

The loss of viability observed after exposure to 0.4 mM ATP was clearly decreased when Pi was omitted from the incubation medium. However, the onset of cell death was delayed and not prevented. This is explained by the fact that a large amount of extracellular phosphate is formed after hydrolysis of the extracellular ATP at the plasma membrane. A similar increase in [Ca\textsuperscript{2+}] as with ATP, causing a comparable rate of cell killing, could be obtained by exposing the cells to 1 mM ATP\textsubscript{yS}, a nonhydrolyzable analog of ATP. In this case omission of phosphate from the medium, which now cannot be compensated by P\textsubscript{i} generated from hydrolysis, provided total protection against cell death.

Exposure to ATP or ATP\textsubscript{yS} caused a large net uptake of phosphate by the cells. The increased uptake of phosphate was reflected by an increased cellular P\textsubscript{i} content. To our knowledge this is the first report about manipulation of cellular P\textsubscript{i} after treatment of hepatocytes with any agent, without depletion of intracellular ATP. On the contrary, ATP, also greatly increased. Earlier work by our group revealed that the increase in [ATP], is dependent on the induced increase in [Ca\textsuperscript{2+}], and not caused by uptake of extracellular ATP (20).

When P\textsubscript{i} was omitted from the medium the induced synthesis of [ATP], was markedly delayed, probably due to a lesser phosphate availability for synthesis caused by a lower uptake of phosphate from the medium. On the other hand, lower cellular P\textsubscript{i} levels possibly reduce a Ca\textsuperscript{2+}-induced stimulation of mitochondrial respiration by interfering with mitochondrial Ca\textsuperscript{2+} transport.

In isolated mitochondria phosphate facilitates Ca\textsuperscript{2+} uptake causing high matrix Ca\textsuperscript{2+} levels which induce permeability changes of the inner membrane. In cultured myocytes mitochondrial Ca\textsuperscript{2+} levels were not very different from cytosolic Ca\textsuperscript{2+} levels (28). In our freshly isolated hepatocytes higher calcium ratios (which reflected at least a two times higher free calcium concentration) were measured in mitochondria than in the whole cell. Nevertheless, in intact cells accumulation of free calcium in the mitochondria prior to cell death was measured after induction of high [Ca\textsuperscript{2+}], by extracellular ATP. This accumulation was reduced when an increase in intracellular P\textsubscript{i} content was less.

The data presented in this paper are in agreement with the hypothesis that mitochondrial dysfunction and cell death in intact hepatocytes observed after an ATP-induced, sustained increase in [Ca\textsuperscript{2+}], is associated with increased mitochondrial Ca\textsuperscript{2+} levels. The mitochondrial uptake of Ca\textsuperscript{2+} is dependent on intracellular P\textsubscript{i}. After exposure to ATP the requirements for permeability changes of the mitochondrial inner membrane, i.e. high mitochondrial matrix calcium and the presence of a large amount of phosphate as inducing agent, are present. Permeabilization of the mitochondrial inner membrane is most likely involved in ATP-induced mitochondrial dysfunction and cell death. The exact mechanism of the permeability transition is not yet completely understood. Several processes, such as activation of Ca\textsuperscript{2+}-dependent phospholipases (43) or ADP ribosylation (1), have been proposed to be responsible for the nonspecific pore opening in the mitochondrial inner membrane.

It has been suggested that activation of Ca\textsuperscript{2+}-dependent proteases is involved in Ca\textsuperscript{2+}-induced cell killing (44). However, several groups could not reproduce any protective effect of protease inhibitors on cell death in hepatocytes in which calcium was elevated (15, 20, 45, 46). The present results and previous work from our laboratory strongly indicate that a calcium-induced permeability transition of the mitochondrial inner membrane plays an important role in calcium-dependent cytotoxicity in hepatocytes.

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$P_{i}$-dependent Ca$^{2+}$-induced Cell Killing by ATP

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