Short Communication

Tetraphenylphosphonium-Selective Electrode as a Tool for Evaluating Mitochondrial Permeability Transition Pore Function in Isolated Rat Hepatocytes

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Abstract. The changes in mitochondrial membrane potential ($\Delta\psi_m$) were used as an indicator for evaluating the mitochondrial permeability transition pore (MPTP) function. We found that *in situ* mitochondria in digitonin-permeabilized hepatocytes were coupled and responded to the addition of substrates, inhibitors and uncouplers. Ca^{2+}-induced $\Delta\psi_m$ dissipation was caused by MPTP opening because this process was inhibited by cyclosporin A. MPTP opening was enhanced by the pro-oxidant *tert*-butyl hydroperoxide.

Key words: Tetraphenylphosphonium-selective electrode — Mitochondrial permeability transition pore — Hepatocytes

Abbreviations: $\Delta\psi_m$, mitochondrial membrane potential; MPTP, mitochondrial permeability transition pore; TPP^+, tetraphenylphosphonium; ADP, adenosine diphosphate; *t*-BHP, *tert*-butyl hydroperoxide; FCCP, carbonyl cyanide p-trifluoro-methoxyphenylhydrazone; CsA, cyclosporin A; EGTA, ethylene glycol-bis-(β-aminoethylether) tetraacetic acid.

It is now generally accepted that mitochondria are involved in cell apoptosis and that the opening of the mitochondrial permeability transition pore (MPTP) initiates the process of cell death (Halestrap et al. 2002; Di Lisa and Bernardi 2006). The participation of the MPTP is involved in both apoptotic and necrotic cell killing and it is associated with hypoxia and ischemia-reperfusion-induced oxidative stress,

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with acceleration of ageing processes (Lemasters et al. 1998), with various neurodegenerative diseases (Shapira 1994), and with chemical toxicity effects (Quian et al. 1999).

In spite of this general consensus, the mechanism of the MPTP function and the role of many factors participating in MPTP regulation are not yet fully clarified because the number of various enzymes and proteins participating directly or indirectly in MPTP function as well as the number of various factors, endogenous and exogenous, that modify its function is continuously increasing (Halestrap et al. 2002; Di Lisa and Bernardi 2006).

MPTP was discovered and studied on isolated mitochondria and mitochondrial swelling. Changes of Ca^{2+} transport, and mitochondrial membrane potential ($\Delta\psi_m$) were used as a methodological tool (Halestrap et al. 2002; Di Lisa and Bernardi 2006). However, some data have recently appeared indicating that mitochondrial contacts with endoplasmic reticulum and other cell structures as well as with other mitochondria are important for mitochondrial functional activity (Skulachev 2001; Garesse and Vallejo 2001). Therefore, more attention should be paid to studies on intact cells. Isolated hepatocytes, permeabilized by digitonin, can be used as a useful biological model because the intracellular network is maintained (Fiskum et al. 1980) and internalized mitochondria are accessible for exogenous substrates and cofactors.

Our previous studies have been focused on the mechanisms that participate in the oxidative damage of liver cells by various hepatotoxic agents (Drahota et al. 2005; Ferenčíková et al. 2003; Ješina et al. 2004). Because peroxides facilitate MPTP opening (Halestrap et al. 2002), we used the tetraphenylphosphonium (TPP^+)-selective electrode (Labajova et al. 2006) for evaluating the changes in $\Delta\psi_m$ and we used this technique for assessing the MPTP function under oxidative stress. We tested to which extent MPTP participates in oxidative damage induced by the pro-oxidant *tert*-butyl hydroperoxide (*t*-BHP).

We used rat hepatocytes prepared according to Farghali et al. (1994). The viability of cells was tested by Tryptan Blue Dye exclusion and preparations with viability higher than 90% were used. Respiration of hepatocytes was measured by high resolution Oxygraph-2k OROBOROS (Austria) at $30°C$ in KCl medium containing (in mmol/l): 100 KCl, 10 Tris-HCl, 3 $MgCl_2$, 4 KH_2PO_4 and 1 EDTA (pH 7.4.) OROBOROS software DatLab 3 was used for calculating the respiratory rates and for presentation of oxygen consumption curves. $\Delta\psi_m$ was evaluated as changes of TPP^+ concentration in the incubating medium (Labajova et al. 2006), a decrease in TPP^+ concentration indicating TPP^+ uptake by the energized mitochondria. Calibration was done before each measurement. A computerized device for membrane potential measurement contained the TPP^+-selective electrode, a reference Ag/AgCl electrode, and PC with the high impedance measuring card PCI-6036 (National Instruments, USA). The TPP^+-selective membrane was prepared as described before (Labajova et al. 2006). Data acquisition, filtration, and storage was done using MATLAB/Simulink software (MathWorks, Inc., USA). The membrane potential measurements were performed at $25°C$ in KCl medium with-
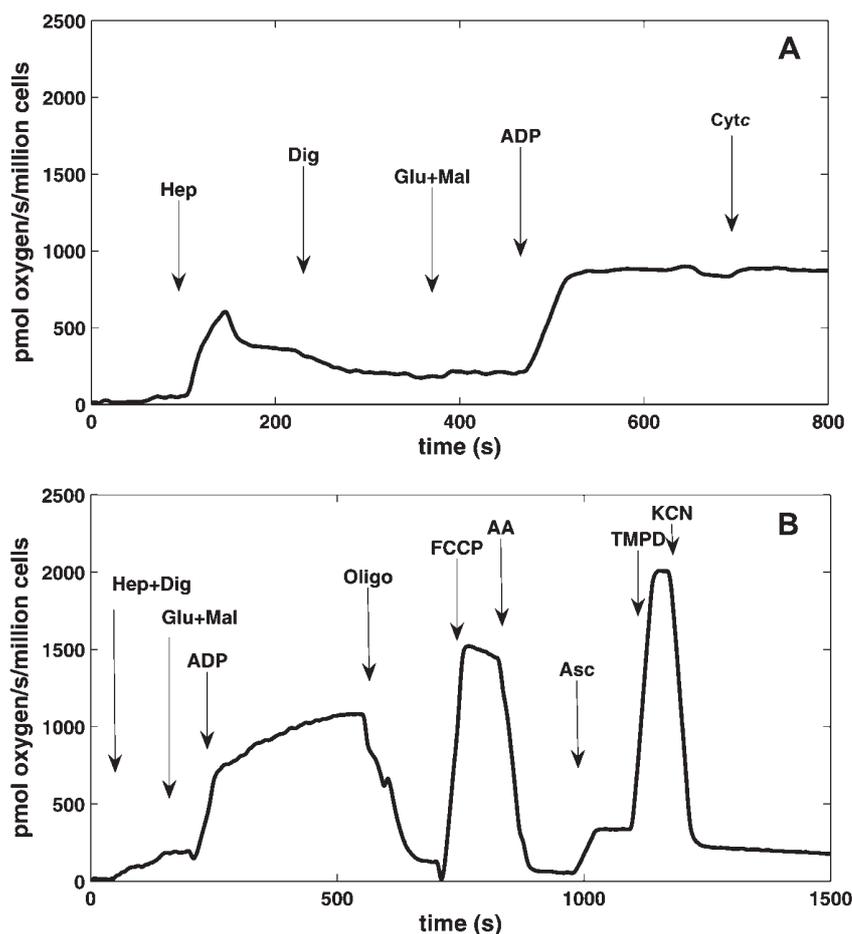
Figure 1. Respiration of hepatocytes permeabilized by digitonin. All experiments were repeated on three different cell preparations with the same results. **A.** Hepatocytes (Hep) were incubated at 30 °C in KCl medium. Where indicated, Hep (0.25 million cells/ml), digitonin (Dig, 20 μg *per* ml), glutamate + malate (Glu+Mal, 10 + 3 mmol/l), adenosine diphosphate (ADP, 1.5 mmol/l), cytochrome *c* (Cyt*c*, 20 μmol/l were added. **B.** Experimental conditions were as in (A). Where indicated, Glu+Mal (10 + 3 mmol/l), ADP (1.5 mmol/l), oligomycin (Oligo, 2 μmol/l), carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP, 1 μmol/l), antimycin A (AA, 1 μmol/l), ascorbate (Asc, 2 mmol/l), tetra methyl p-phenylene diamine (TMPD, 0.5 mmol/l) or KCN (0.5 mmol/l) were added. The curves represent first negative derivation of oxygen tension changes in the incubation medium. Oxygen uptake is expressed as pmoles oxygen *per* second *per* million cells.

out ethylene diamine tetraacetic acid (EDTA). Where indicated, 1 mmol/l EDTA was added.

For the detection of membrane potential changes we used hepatocytes per-

meabilized by digitonin. Under these experimental conditions there was free access of respiratory substrates and adenosine diphosphate (ADP) to cytosol and the internalized mitochondria were well coupled (Fig. 1A). The respiratory control index (–ADP/+ADP) was 4.3. The addition of cytochrome c had no activating effect on the respiratory rate, which confirms that the outer mitochondrial membrane remains intact at the digitonin concentration used. Fig. 1B demonstrates that the internalized mitochondria responded to an added uncoupler – carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) and respiratory-chain inhibitors (oligomycin, antimycin A, KCN) like isolated mitochondria.

It is known that MPTP opening is caused by Ca^{2+} overload followed by dissipation of $\Delta\psi_m$ and consequent mitochondrial swelling. However, $\Delta\psi_m$ dissipation can also occur due to various uncouplers (FCCP, CCCP, free fatty acids). Therefore, cyclosporin A (CsA) – an inhibitor of MPTP should be used to prove that $\Delta\psi_m$ dissipation is caused by MPTP opening.

When we tested changes of the $\Delta\psi_m$ in hepatocytes permeabilized by digitonin and energized by succinate, we found that the membrane potential is maintained only for a short period after digitonin addition (about 4 min) and then quickly dissipates (Fig. 2A). This dissipation can be reversed by the addition of ethylene glycol-bis-(β-aminoethylether) tetraacetic acid (EGTA) (Fig. 2A) or it can be completely prevented by CsA (Fig. 2B) which indicates involvement of Ca^{2+}-activated CsA-sensitive MPTP function. The effect of calcium ions was concentration-dependent and in agreement with previous findings (Halestrap et al. 2002) at higher Ca^{2+} concentrations (above 250 μmol/l Ca^{2+}), $\Delta\psi_m$ dissipated even in the presence of CsA (Fig. 2B). Addition of EGTA again restored the membrane potential. The value of $\Delta\psi_m = 0$ is represented by TPP^+ concentration after FCCP addition (Figs. 2 and 3). The difference between original TPP^+ concentration and concentration after FCCP addition indicates nonspecific binding to the cell structures and also TPP^+ dilution due to the volume change after hepatocyte permeabilization. These findings show that Ca^{2+} present in the medium during hepatocyte isolation cannot be completely removed by repeated washings, or that hepatocytes are sufficiently preloaded by Ca^{2+} during the isolation procedure to open the MPTP after digitonin addition.

Therefore, in further experiments, when we tested the effect of t-BHP, we used medium with 1 mmol/l EDTA that completely prevented dissipation of the membrane potential by contaminating Ca^{2+} ions (Fig. 3A). Under these experimental conditions, the membrane potential was not affected by Ca^{2+} added to concentrations below 200 μmol/l (Fig. 3A). Also t-BHP up to 1.5 mmol/l in the medium with 1 mmol/l EDTA had no dissipating effect (Fig. 3B). However, when 1.5 mmol/l t-BHP was added in the presence of 50 μmol/l Ca^{2+}, membrane potential was dissipated (Fig. 3C) and the dissipation was prevented by CsA (Fig. 3D). This confirms previous observations indicating that oxidative stress increases the sensitivity of MPTP to Ca^{2+} (Halestrap et al. 2002).

We may thus conclude that our device for measuring the $\Delta\psi_m$ changes can be used for the evaluation of hepatotoxic action of various agents that damage
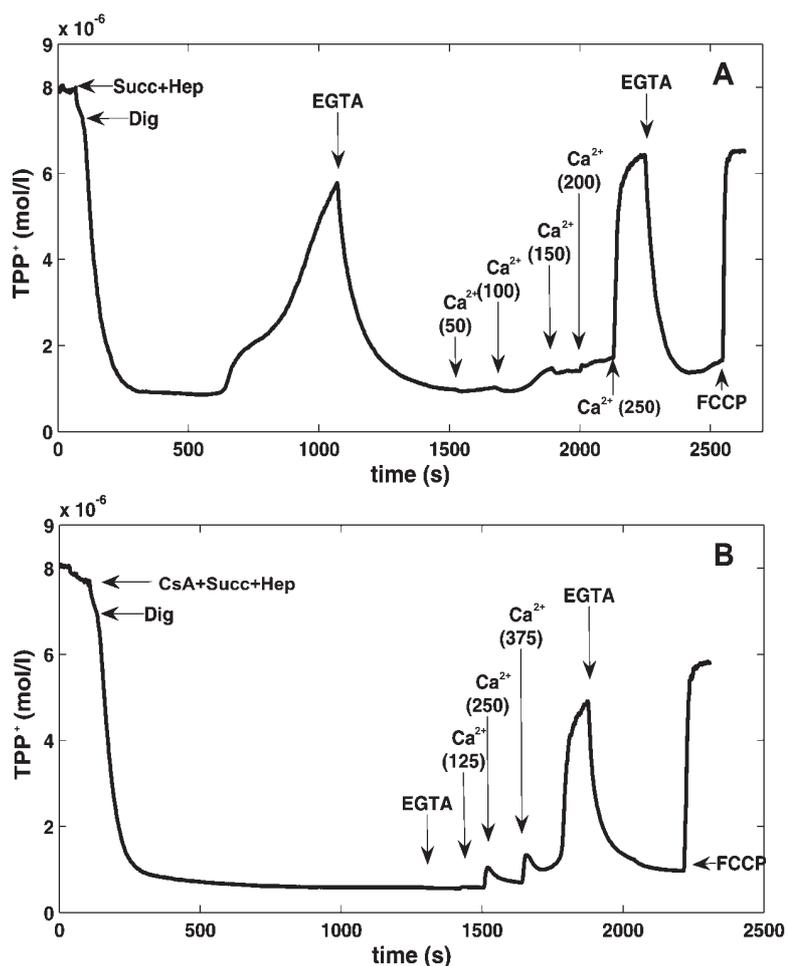
Figure 2. Changes of tetraphenylphosphonium (TPP$^+$) concentration in the medium induced by calcium and ethyleneglycol-bis-(β-aminoethylether) tetraacetic acid (EGTA). Hepatocytes (Hep) were incubated at 25 °C in KCl medium without EDTA. All experiments were repeated on three different cell preparations with the same results. Arrows indicate additions to the final concentration of: **A.** succinate (Succ, 10 mmol/l), Hep (1.64 million cells/ml), digitonin (Dig, 0.035 mg/ml), EGTA (350 μmol/l), Ca^{2+} (50, 100, 150, 200, 250 μmol/l), EGTA (3 mmol/l), carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP, 1 μmol/l); **B.** cyclosporin A (CsA, 2 μmol/l), Succ (10 mmol/l), Hep (1.64 million cells/ml), Dig (0.035 mg/ml), EGTA (350 μmol/l), Ca^{2+} (125, 250, 375 μmol/l), EGTA (3 mmol/l), FCCP (1 μmol/l).
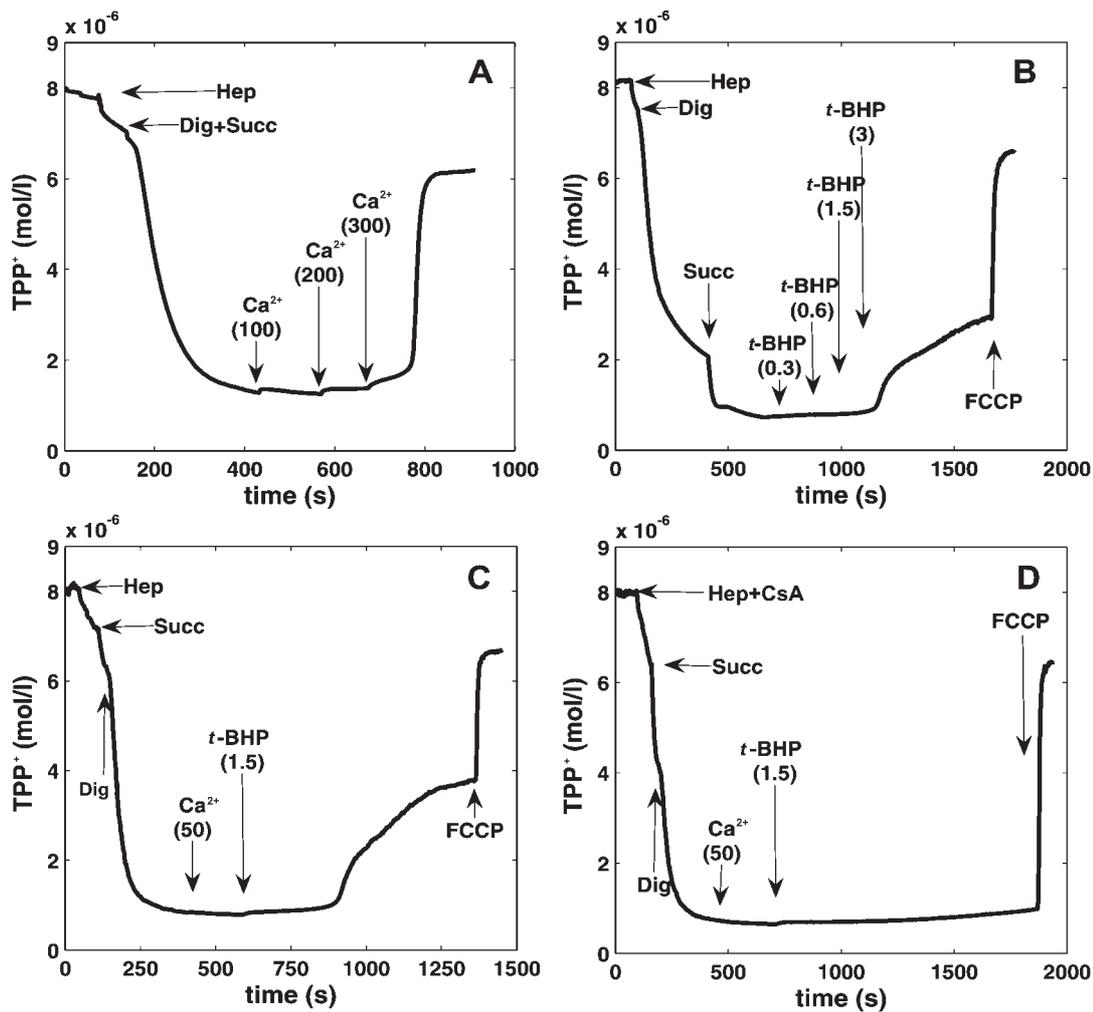
Figure 3. Changes of tetraphenylphosphonium (TPP^+) concentration in the medium induced by calcium and *tert*-butyl hydroperoxide (*t*-BHP). Hepatocytes (Hep) were incubated at 25 °C in KCl medium with 1 mmol/l EDTA. All experiments were repeated on three different cell preparations with the same results. Arrows indicate addition to the final concentration of: **A.** succinate (Succ, 10 mmol/l), Hep (1.8 million cells/ml), digitonin (Dig, 0.035 mg/ml), Ca^{2+} (100, 200, 300 mol/l); **B.** Succ (10 mmol/l), Hep (1.8 million cells/ml), Dig (0.035 mg/ml), *t*-BHP (0.3, 0.6, 1.5, 3 mmol/l), carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP, 1 μmol/l); **C.** Succ (10 mmol/l), Hep (1.64 million cells/ml), Dig (0.035 mg/ml), Ca^{2+} (50 μmol/l), *t*-BHP (1.5 mmol/l), FCCP (1 μmol/l); **D.** Hep (1.64 million cells/ml), cyclosporin A (CsA, 2 μmol/l), Succ (10 mmol/l), Dig (0.035 mg/ml), Ca^{2+} (50 μmol/l), *t*-BHP (1.5 mmol/l), FCCP (1 μmol/l).

hepatocyte energy metabolism due to the activation of MPTP opening. According to the recent findings (Halestrap et al. 2002; Di Lisa and Bernardi 2006), MPTP opening is the first step in the development of apoptotic and necrotic processes.

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