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Evaluation of mitochondrial membrane potential using a computerized device with a tetraphenylphosphonium-selective electrode

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Abstract

Mitochondrial membrane potential ($\Delta\psi_m$) plays important roles in the normal function of cells and in pathobiochemical situations. The application of ion-selective electrodes for the measurement of $\Delta\psi_m$ is important for studying normal biological reactions and pathways and mitochondrial diseases. We constructed and optimized a computerized device for real-time monitoring of the $\Delta\psi_m$, which included modification of tetraphenylphosphonium (TPP^+)-selective membrane that improved reproducibility of the TPP^+-selective electrode. Application of MATLAB software increased the sensitivity of the system. We tested our improved device for membrane potential measurements of isolated mitochondria (in absolute scale of millivolts). In addition, we assessed relative changes of $\Delta\psi_m$ (as changes in TPP^+ concentration) of digitonin-permeabilized cells (hepatocytes, control transmitochondrial cybrids, HeLa G and BSC-40) after addition of substrates, inhibitors, and uncoupler of respiratory chain. Our system can be successfully used for studies of many aspects of the regulation of mitochondrial bioenergetics and as a diagnostic tool for mitochondrial oxidative phosphorylation disorders.

Keywords: Membrane potential; TPP-selective electrode; Mitochondria; Hepatocytes; Cultured cells

Mitochondria are the main energy producers in eukaryotic cells. Therefore, their functional defects, induced either by genetic mutation [1] or by numerous toxic factors [2,3], play important roles in the pathogenesis of many diseases. The key role of mitochondria is synthesis of ATP from ADP and phosphate, which may represent up to 90% of all ATP produced in cells and tissues with high aerobic metabolism. This process is driven by the electrochemical potential generated by the respiratory chain enzymes (complexes I, III, and IV) located in the inner mitochondrial membrane. Transfer of electrons from substrates to oxygen provides energy for pumping of protons across the membrane and thus generates electrochemical potential ($\Delta\tilde{\mu}_{H^+}$) described by the equation

$$\Delta\tilde{\mu}_{H^+} = -2.3RT\Delta pH + F\Delta\psi_m, \quad (1)$$

where ΔpH is the pH difference and $\Delta\psi_m$[1] is the mitochondrial membrane potential across the mitochondrial membrane, negative inside. $\Delta\psi_m$ forms the major component of the electrochemical potential and therefore is a convenient parameter for the evaluation of mitochondrial function.

For measurement of the membrane potential, lipid-soluble cations and anions are widely used. These ions include fluorescent probes (rhodamine 123, tetramethylrhodamine methyl ester, JC-1 [4]), radiolabeled probes ([^{14}C]tetraphenylphosphonium, [^{3}H]methyltriphenylphosphonium [5]), and unlabeled probes (tetraphenylphosphonium (TTP^+),

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[1] *Abbreviations used:* $\Delta\psi_m$, mitochondrial membrane potential; TPB^-, tetraphenylboron; TPP^+, tetraphenylphosphonium; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; DDA^+, dibenzyldimethyl ammonium; EMF, electromotive force; PVC, polyvinyl chloride.

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dibenzyldimethyl ammonium (DDA^+)) [6,7]. They permeate cell membranes [8,9] and distribute between cells/organelles and their surroundings in accordance with the Nernst equation,

$$\Delta\psi = \frac{RT}{zF}\ln\frac{a_{out}}{a_{in}}, \quad (2)$$

where R, T, z, and F are the universal gas constant, the absolute temperature, the valence, and the Faraday constant, respectively, and a_{out} and a_{in} are the activities of the lipid-soluble ions inside the cell/organelle and in the medium. The concentration of unlabeled probe (ion activity) in the medium can be determined by an ion-selective electrode. This allows determining the changes of the probe accumulation and thus the membrane potential continuously and in the absolute scale of millivolts. Shinbo et al. [7] used DDA^+ as the membrane potential indicator. It was soon replaced by TTP^+ because it permeates membranes about 15 times faster than DDA^+ and does not require the lipid-soluble anion tetraphenylboron in the incubation medium [6]. This approach was widely used and TPP^+ electrodes have Nerstian response, but their reproducibility is not fully satisfying.

Ion-selective electrodes are typically employed under zero-current condition in a galvanic cell which consists of an ion-selective electrode, a reference electrode (Ag/AgCl electrode or Hg/Hg_2Cl_2 electrode), aqueous medium with biological sample, and a device for electromotive force (EMF) monitoring. EMF across the galvanic cell is the sum of all individual contributions:

$$\mathrm{EMF} = E_{const} + E_J + E_M. \quad (3)$$

E_M is the membrane potential, E_J is the liquid junction potential at the sample/bridge electrolyte interface, and E_{const} is the sample-independent constant. If the membrane internal diffusion potential is zero and the ion activity of the primary ion in the membrane does not change, E_M is described by the Nernst equation,

$$E_M = E^0 + \frac{RT}{zF}\ln(a_i)_s, \quad (4)$$

where E^0 denotes sample-independent variables and $(a_i)_s$ is the ion activity of the primary ion in the sample solution. In real measurements, the electrode can respond to various ions present in the medium other than the primary ions. These ions are called interfering ions. If interference occurs, the semiempirical Nicolskii–Eisenman equation [10] is used for description of the ion-selective electrode response,

$$E_M = E^0 + \frac{RT}{z_iF}\ln(a_i(ij) + K_{ij}^{pot}a_j(ij)^{z_i/z_j}), \quad (5)$$

where $a_i(ij)$ and $a_j(ij)$ are the activities of the primary ion i and interfering ion j in the mixed solution, K_{ij}^{pot} is the Nicolskii coefficient, z_i is the valence of the ion i, and z_j is the valence of the interfering ion j.

In this paper, we show the construction details of the optimized system for the membrane potential measurement based on TPP^+-selective electrode. We describe the preparation of the TPP^+-selective electrode and TPP^+-selective membrane and optimization of their properties. We also describe the protocol for membrane potential measurements of isolated rat liver mitochondria and mitochondria of isolated hepatocytes and cultured cells permeabilized by digitonin.

Materials and methods

Reagents

Tetraphenylphosphonium (TPP^+Cl^-, TPP^+Br^-), tetraphenylboron (Na^+TPB^-), substrates of respiratory chain (pyruvate, malate and succinate), and inhibitors of respiratory chain (rotenone and FCCP) were obtained from Sigma–Aldrich (USA). Digitonin was obtained from Fluka (USA).

Isolation of rat liver mitochondria and hepatocytes

Two-month-old male Wistar rats (Velaz Lysolaje, Czech Republic) with initial body weights of 180–220 g were used. Animals were housed at 23 ± 1 °C with a 12 h:12 h light/dark period and had free access to standard laboratory rat chow and water. Animals were killed by decapitation in Narcotan narcosis. All animal studies were approved by the animal care committee of the Institute of Physiology and fulfilled HIH guidelines for the human use of animal subjects. Rat liver mitochondria were isolated from overnight-starved rats by differential centrifugation in a sucrose medium (0.25 mM sucrose, 10 mM Tris–HCl, 1 mM EDTA, pH 7.4) and suspended in the same medium as described before [11]. The amount of mitochondrial protein was determined according to Bradford [12]. Rat hepatocytes were isolated in Krebs–Henseleit medium as described by Farghali et al. [13].

Cell cultures

The control transmitochondrial cybrids [14] and HeLa G and BSC-40 cells were cultured in Dulbecco's modified Eagle's medium (SEVAC, Czech Republic) with 10% fetal calf serum (Sigma, USA) at 37 °C in 5% CO_2 in air. Confluent cells were harvested using 0.05% trypsine and 0.02% EDTA. Detached cells were diluted with ice-cold cultured medium, sedimented by centrifugation and washed twice in cold phosphate-buffered saline.

High-resolution respirometry

The oxygen consumption was measured by Oxygraph-2k (Oroboros, Austria) in a K medium (80 mM KCl, 10 mM Tris–HCl, 3 mM $MgCl_2$, 5 mM KH_2PO_4, and 1 mM EDTA, pH 7.4) at 30°C. The data were analyzed with DatLab2 software (Oroboros, Austria); the rates of oxygen consumption of isolated mitochondria were

normalized on protein content and expressed as pmol s^{-1} mg^{-1} protein. For permeabilized cells the rate of oxygen consumption was expressed as pmol s^{-1} 10^{-6} cells [15].

Results and discussion

Construction of the device for membrane potential measurements

The computerized device for membrane potential measurement was constructed in our laboratory. It is composed of the measuring chamber (maximum sample volume 5 ml, open top system allowing for addition of reagents during measurements) with the magnetic stirrer, the reference electrode, the TPP^+-selective electrode, and PC which includes the high-impedance measuring card PCI-6036E (National Instruments, USA) (Fig. 1). Signal acquisition, processing, and display were realized by MATLAB/Simulink software (The MathWorks, Inc., USA). The Simulink modules execute real-time acquisition, signal filtration, and data storage under the Windows operating system. The signal filtration significantly increases the signal/noise ratio. The system was placed into the Faraday cage to shield against electromagnetic radiation.

We chose Plexiglas and Teflon as materials suitable for the electrode body and the measuring chamber because of their physical properties. For details of the electrode construction see Fig. 1B. The TPP^+-selective membrane was glued to the electrode body using tetrahydrofuran and fixed with a Plexiglas ring, which has an inner conical shape to ensure constant tension of the PVC membrane in contrast to silicon O rings.

Preparation of the TPP^+-selective membrane

We prepared the PVC-based TPP^+-selective membrane according to Kamo et al. [6]. The solution A (619.12 mg PVC, 12.38 ml tetrahydrofuran, and 1.44 ml dioctyl phthalate) and the solution B (24.77 mg tetraphenylboron and 4.95 ml tetrahydrofuran) were mixed and poured into a petri dish (diameter 89 mm), followed by slow evaporation at room temperature. Three different types of PVC made in the Czech Republic were tested: Neralit 581, Neralit 682, and Neralit 702 (Spolana, Neratovice). The most suitable was Neralit 702 because of its elasticity and solidity.

We also compared the response of the electrodes which were filled either with 10 mM TPP^+Cl^- or with 10 mM TPP^+Br^- as the inner filling solution. The electrode filled with TPP^+Cl^- had non-Nerstian response in contrast to the electrode filled with TPP^+Br^-. However, the response of the latter electrode was not fully reproducible after changing the membrane. To improve it, we modified the membrane preparation according to Shinbo et al. [7]. Instead of Na^+TPB^- we added the precipitate of TPP^+Cl^- and Na^+TPB^- to the solution B. The modified TPP^+-selective membrane had the Nernstian response to TPP^+ from 3 μM TPP^+ (Fig. 2A) which was independent of the filling solution (10 mM TPP^+Cl^- or TPP^+Br^- was used).

The electrode with new prepared membrane had stable and reproducible response for 2 to 3 months. After each measurement the electrode should be rinsed several times with water. When rotenone or other inhibitors are used the electrode should be rinsed with ethanol. The electrode is stored in dry place and before measurement it has to be soaked in 10 mM TPP^+Cl^-.

Such modified TPP^+-selective electrode filled with TPP^+Cl^- was used for the measurements of the mitochondrial membrane potential.

Determination of the membrane potential of isolated rat liver mitochondria and mitochondria in isolated and cultured cells: the effect of substrates and inhibitors

Using our system we tested changes of the membrane potential of isolated mitochondria and mitochondria in cells. To quantify the amount of TPP^+ accumulated in the mitochondria, it is necessary to calibrate the electrode by successive additions of TPP^+ (Fig. 2B) before each measurement. The measured data plotted in logarithmic scale of TPP^+ concentration are shown in the inset of Fig. 2B.

Changes of the mitochondrial membrane potential are often presented as the changes of TPP^+ concentration in

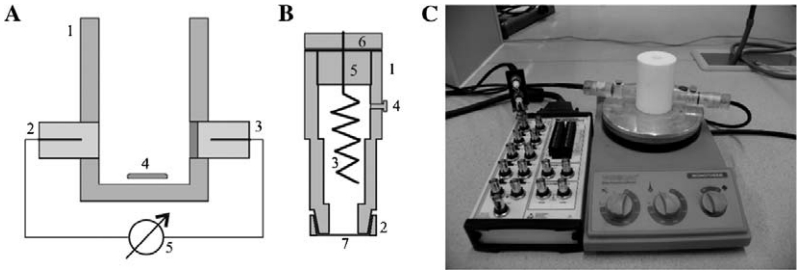

Fig. 1. (A) Schematic diagram of the membrane potential measuring circuit: 1, the measuring chamber; 2, the reference Ag/AgCl electrode; 3, the TPP^+-selective electrode; 4, the magnetic stirrer; 5, PC with PCI-6036E measuring card. (B) Construction details of TPP^+-selective electrode: 1, the electrode body; 2, the Plexiglas ring; 3, the Ag/AgCl wire; 4, the opening for inner electrolyte filling; 5, the Plexiglas block where the Ag wire is sealed; 6, the Plexiglas ring with the tread to fasten the Plexiglas block inside the electrode body; 7, PVC membrane selective to TPP^+. (C) Photograph of electrode assembly.

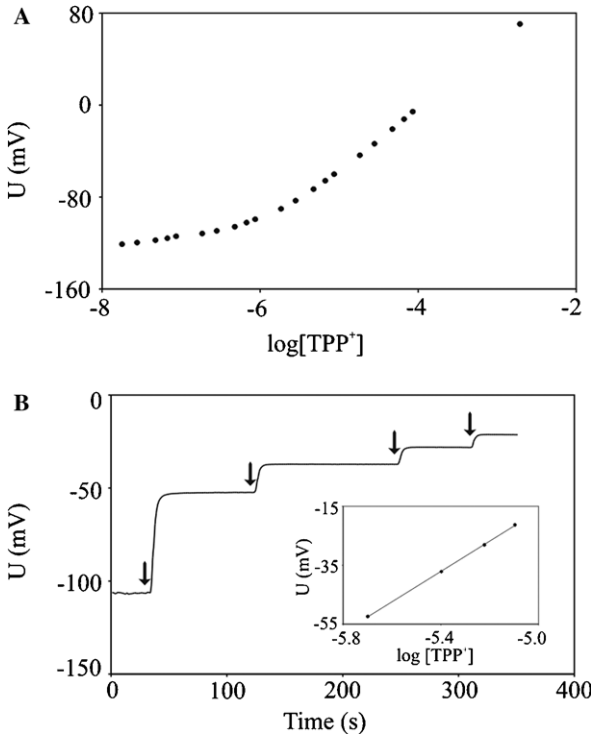

Fig. 2. Response of the electrode to TPP^+ with the membrane prepared by modified procedure. (A) Calibration of TPP^+-selective electrode by successive addition of TPP^+ to 0.1 M NaCl in the concentration range 20 nM–2 mM. (B) Calibration of TPP^+-selective electrode in concentration range 2–8 μM TPP^+. Arrows indicate additions of 4 μl of 10 mM TPP^+. (Inset) Electrode response as a function of logarithm of TPP^+ concentration.

the incubation medium. The value of $\Delta\psi_m$ of isolated mitochondria can be calculated using the equation

$$\Delta\psi_m = \frac{RT}{F}\ln\frac{V_0[TPP^+]_0/[TPP^+]_t - V_t - K_0P}{V_mP + K_iP}, \quad (6)$$

where V_0 is the volume of the medium before mitochondria addition, V_t is the final volume, V_m is the volume of mitochondrial matrix (μl/mg protein), and $[TPP^+]_0$ and $[TPP^+]_t$ are the concentrations of TPP^+ prior to addition of mitochondria and at time t, respectively. P is the mitochondrial protein content in the chamber (mg); K_0 (14.3 μl/mg) and K_i (7.9 μl/mg) are apparent external and internal partition coefficients of TPP^+, respectively [16]. The volume of mitochondria was taken as 1 μl/mg of protein [17].

The analysis of the changes in $\Delta\psi_m$ (as changes of TPP^+ concentration) of isolated rat liver mitochondria (Fig. 3) was performed in a K medium that is used also for oxygraphic analysis (80 mM KCl, 10 mM Tris–HCl, 3 mM $MgCl_2$, 5 mM KH_2PO_4, and 1 mM EDTA, pH 7.4). After addition of mitochondria the TPP^+ concentration in the medium decreased due to TPP^+ uptake into the mitochondrial matrix. The following slow efflux of TPP^+ into the medium indicated the decrease of $\Delta\psi_m$ owing to depletion of endogenous substrates. Pyruvate and malate addition (the substrates of NADH-dependent dehydrogenases) prevented the TPP^+ concentration

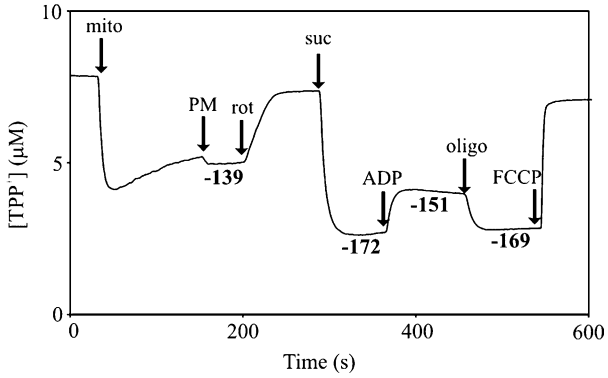

Fig. 3. Analysis of $\Delta\psi_m$ of isolated rat liver mitochondria. Arrows indicate the addition of 0.3 mg/ml of isolated mitochondria (mito), 10 mM pyruvate (P), 2.5 mM malate (M), 1 μM rotenone (rot), 10 mM succinate (suc), 0.5 mM ADP, 1 μM oligomycin (oligo), and 1 μM FCCP. Appropriate values of the mitochondrial membrane potential in millivolts are shown.

increase. Addition of rotenone induced dissipation of $\Delta\psi_m$ due to its inhibition of complex I. Subsequent addition of succinate (substrate of complex II) again restored $\Delta\psi_m$ to values even higher than those obtained in the presence of pyruvate and malate. To test the function of ATP synthase that utilizes the proton gradient for ATP synthesis, ADP was added, which caused the release of TPP^+, indicating a partial depolarization. Oligomycin, specific inhibitor of ATP synthase, restored $\Delta\psi_m$ and the uncoupler FCCP decreased $\Delta\psi_m$ to a minimum value. The values of $\Delta\psi_m$ that were calculated using Eq. (6) are included in Fig. 3. The obtained data are in accordance with the previously published studies [18,19]. In parallel experiments on the oxygraph we confirmed that mitochondria used for measurements were tightly coupled, with a respiratory control index of 4–6 (not shown).

The calculation of $\Delta\psi_m$ in intact cells is more complicated because TPP^+ accumulation is controlled by both the plasma membrane potential and the mitochondrial membrane potential. Moreover, $\Delta\psi_m$ calculation requires the values of the mitochondrial volume inside the cells and the amount of TPP^+ that binds nonspecifically to the various cell membranes. For that reason, we permeabilized the cells by digitonin (Fluka, USA) to collapse the plasma membrane potential and then used the changes of TPP^+ concentration as an indicator of relative changes of $\Delta\psi_m$. The determination of $\Delta\psi_m$ in situ in permeabilized cells has some advantages because during the isolation some of the mitochondria may be lost or destroyed and only a fraction of the original mitochondria is isolated. Such mitochondria also lose their interorganelle contacts which may affect their function [20,21]. Fig. 4A shows TPP^+ concentration changes using hepatocytes upon their permeabilization with 0.35 mg/ml of digitonin and subsequent addition of substrates and inhibitors of the respiratory chain. Measurements were performed again in the mitochondrial K medium. Only small TPP^+ accumulation occurred after addition of hepatocytes. Addition of digitonin caused collapse of the plasma membrane potential and

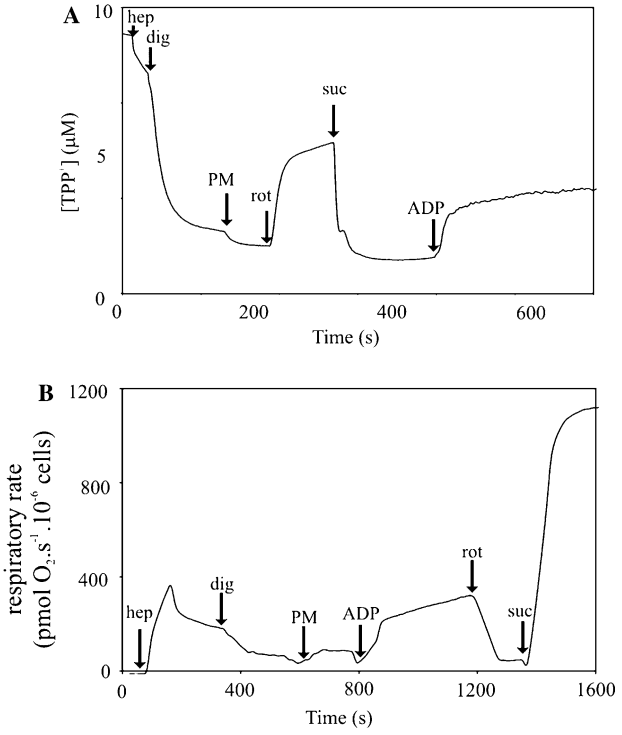

Fig. 4. Analysis of $\Delta\psi_m$ (A) and respiration (B) of isolated hepatocytes. Arrows indicate the addition of 1.85 mil/ml of rat liver hepatocytes (hep), 0.35 mg/ml of digitonin (dig), 10 mM pyruvate (P), 2.5 mM malate (M), 1 mM ADP, 1 μM rotenone (rot), and 10 mM succinate (suc).

pronounced decrease of TPP^+ concentration in the medium due to the probe accumulation in mitochondria. The changes of $\Delta\psi_m$ after subsequent addition of substrates and inhibitors were similar to $\Delta\psi_m$ changes of isolated mitochondria (Fig. 4A). Parallel experiments on the oxygraph showed that under similar conditions the mitochondria in permeabilized hepatocytes were coupled and the changes after addition of substrates or inhibitors corresponded to the measurements of $\Delta\psi_m$ (Fig. 4B). This approach proved to be useful also for assessing mitochondrial function in other types of cultured cells such as cybrids, HeLa G or BSC-40 cells where changes of $\Delta\psi_m$ similar to those in hepatocytes were observed (data not shown).

In our hands, the following amounts of the sample per ml of medium were needed for good response measurement: 0.3–0.5 mg protein of liver mitochondria, $1.5–2.0 \times 10^6$ of hepatocytes, and three to fivefold higher amounts of different types of human cultured cells. The optimal concentration of digitonin (to the point where the TPP^+ influx into the mitochondria is independent on the digitonin added) has to be tested with each cell type.

We may summarize that our computerized system has good stability, sensitivity, and reproducibility and can be successfully used for studies of many aspects of the regulation of mitochondrial bioenergetics. It may be also useful as a diagnostic tool in pathological conditions when changes in mitochondrial membrane potential occur. These may be caused by different types of mitochondrial diseases, either due to insufficient generation of $\Delta\psi_m$, as might be the case of complex I or complex IV defects [22], or due to insufficient discharge of $\Delta\psi_m$, as in ATP synthase defects [23–25].

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