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MEASUREMENT OF THE MEMBRANE POTENTIAL USING ION-SELECTIVE ELECTRODE SENSITIVE TO TETRAPHENYLPHOSPHONIUM

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ABSTRACT BOOKLET OF DOCTORAL THESIS

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CONTENT

ABSTRACT

The computerized device for membrane potential measurement using the tetraphenylphosphonium-selective electrode was constructed. Signal acquisition, processing and data storage were realized by MATLAB/Simulink software. The selective membrane was optimized incorporating TPP^+TPB^- precipitate into the membrane. The electrode had a Nernstian response from $3x10^{-6}$ M TPP^+. The TPP^+TPB^- precipitate was later replaced by sodium tetrakis[3,5-bis(1,1,1,3,3,3-hexafluoro-2-methoxy-2-propyl)phenyl]borate (NaHFPB). The electrode with incorporated NaHFPB had a Nernstian response from $1x10^{-6}$ M TPP^+ and had better sensitivity than commercially available electrodes. The values of selectivity coefficients for K^+, Na^+, Ca^{2+} and Mg^{2+} were calculated.

The device was used for mitochondrial membrane potential measurement of isolated mitochondria and for evaluating the respiratory chain function of digitonin-permeabilized cells (hepatocytes, HeLa G, BSC-40 and control transmitochondrial cybrids). This method was used also for monitoring the mitochondrial permeability transition pore (MPTP) function of isolated mitochondria and permeabilized hepatocytes. MPTP opening was induced by high calcium concentration and the action of calcium was enhanced by pro-oxidant *tert*-butyl hydroperoxide (*t*-BHP). This process was inhibited by cyclosporin A.

We also found that *t*-BHP caused $\Delta\psi_m$ dissipation in permeabilized hepatocytes which could be caused by complex I or mitochondrial aconitase inhibition. In addition, *t*-BHP brought about MPTP opening.

Our data indicate that the constructed device can be successfully used for studies of many aspects of mitochondrial bioenergetics, for evaluation of hepatotoxic action of various agents and as a diagnostic tool for mitochondrial oxidative phosphorylation disorders.

1. INTRODUCTION

Ion-selective electrodes have become the well-established group of sensors in the laboratory. Except the glass pH-electrode, electrodes selective to various ions such as Na^+, K^+, Ca^{2+}, I^- [31] and even protamin [33] were developed. The main advantage of the ion-selective electrodes is their large sensitivity range, usually from 1 to 10^{-6} M.

Ion-selective electrodes are typically employed under zero-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 and a device for electromotive force monitoring (emf). Emf across the galvanic cell is the sum of all individual contributions:

$$\text{emf} = E_{const} + E_D + E_M, \tag{1}$$

E_M is the membrane potential, E_D 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 activity of the primary ion in the membrane does not change, the phase boundary potential at the membrane/sample interface (E) is described by the Nernst equation:

$$E = E^0 + \frac{RT}{zF}\ln(a_i)_s, \tag{2}$$

where E^0 denotes the sample-independent variable; R, T, z and F are the universal gas constant, the absolute temperature, the valence and the Faraday constant, respectively, 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 [34] equation is used for description of the ion-selective electrode response:

$$E = E^0 + \frac{RT}{z_i F}\ln\left(a_i(ij) + K_{ij}^{pot} a_j(ij)^{z_i/z_j}\right), \tag{3}$$

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.

The serious limitation of the Nicolskii-Eisenman equation is, however, that for $z_i \neq z_j$, this equation is inconsistent and in the case of exchanging the indices for i and for j the Nicolskii-Eisenman equation does not give the same analytical expression. For this reason, the new formalism was developed [2]. The general result is expressed as:

$$E = E^0 + \frac{RT}{z_i F}\ln(a_i(ij) + \left(K_{ij}^{pot}\right)^{z_j/z_i} a_i(i)^{1-\left(z_j/z_i\right)} a_j(ij)) . \quad (4)$$

For z_i=1 and z_j=2 the equation (4) is expressed as follows:

$$E = E^0 + \frac{RT}{F}\ln\left(\frac{a_i}{2} + \frac{1}{2}\sqrt{a_i(ij)^2 + 4a_j(ij)(K_{ij}^{pot})^2}\right) . \quad (5)$$

For small interference (ca 10 %), the equation (4) can be replaced by:

$$E = E^0 + \frac{RT}{z_i F}\ln(a_i(ij) + \left(K_{ij}^{pot}\right)^{z_j/z_i} a_i(ij)^{1-\left(z_j/z_i\right)} a_j(ij)) . \quad (6)$$

The selectivity, one of the most important characteristics of the ion-selective electrode, can be determined using two different procedures (according to the IUPAC commission, 1997) [38]: *Fixed interference method* and *Separate solution method.* These two methods give meaningful results only if Nernstian slopes are observed for every ion involved [1]. When the condition of Nernstian response for every ion is not fulfilled, the *Matched potential method* can be applied. But the obtained selectivity coefficients (k_{ij}^{MPM}) depend highly on the experimental conditions [3].

For measurement of the membrane potential, lipid-soluble cations and anions are widely used. These include fluorescent probes (rhodamine 123, tetramethylrhodamine methyl ester, JC-1 [30]), radiolabeled probes ([^{14}C]tetraphenylphosphonium, [^{3}H]methyltriphenylphosphonium [7]) and unlabeled probes (tetraphenylphosphonium (TPP^+), dibenzyldimethyl ammonium (DDA^+) [21, 36]. They are electrophoretically transported across the cell membranes [16, 25] and distributed between cells/organelles and their surroundings in accordance with the Nernst equation

$$\Delta\psi = \frac{RT}{zF}\ln\frac{a_{out}}{a_{in}}, \quad (9)$$

where a_{out} and a_{in} are the activities of lipid-soluble ions inside the cell/organelle and in the medium. The changes of lipid-soluble cation concentration (ion activity) in the medium due to their uptake into the mitochondria can be determined by ion-selective electrodes. This allows calculation of the membrane potential in absolute scale of millivolts. Maratsugu et el. (1977) [26] used DDA^+ as a $\Delta\psi_m$ (mitochondrial membrane potential) indicator and developed the DDA^+-selective electrode. This electrode had a liquid membrane with an incorporated ion-exchanger. The liquid selective membrane was soon replaced by the PVC-based membrane

[36]. As TPP^+ for its transport to the mitochondria did not require tetraphenyl boron in the medium and its transport to mitochondria is 15 times faster than DDA^+, DDA^+ was replaced by TPP^+ and the PVC-based TPP^+-selective electrode was constructed [21]. This approach was widely used and TPP^+-selective electrodes had a Nernstian response, but their reproducibility is not fully satisfying and has to be optimized.

Mitochondria are the main energy producers in eukaryotic cells. Their key role is the synthesis of ATP from ADP and phosphate. 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. The transfer of electrons from substrates to oxygen provides energy for pumping of protons across the membrane and thus generates the electrochemical potential ($\Delta\tilde{\mu}_H$) described by the equation:

$$\Delta\tilde{\mu}_H = -2{,}3RT\Delta pH + F\Delta\psi_m , \tag{8}$$

where ΔpH is the pH difference and $\Delta\psi_m$ is the mitochondrial membrane potential across the mitochondrial membrane.

Besides ATP synthesis, mitochondria are involved in calcium metabolism and in the initiation of apoptotic and necrotic cell death as well. Recently, the participation of the mitochondrial permeability transition pore (MPTP) in cell death is discussed [10, 11, 17]. Opening of this pore is also associated with hypoxia and ischemia-reperfusion-induced oxidative stress, with acceleration of aging processes [24], with various neurodegenerative diseases [35] such as Alzheimer disease [41], and with chemical toxicity effects [32].

Mitochondrial permeability transition (MPT) is characterized by increased permeability of the inner mitochondrial membrane to solutes of molecular mass up to ~ 1500 Da [5], subsequent membrane depolarization and the massive swelling due to the colloidal osmotic pressure exerted by the matrix proteins. Mitochondrial swelling may cause the rupture of the outer mitochondrial membrane and the release of various molecules from the intermembrane space such as cytochrome *c*, which, after binding Apaf-1 in the cytosol, causes the activation of caspase 9, triggering the apoptotic cascade [11, 12]. The cause of the MPT is the opening of a non-specific pore at the inner/outer membrane contact site of mitochondria, known as the mitochondrial permeability transition pore. Its structure is still elusive, but the main candidates which could form a core of the complex are: adenine nucleotide translocator (ANT), voltage activated anion channel (VDAC or porin), cyclophilin D (CyP-D). Creatine

kinase, VDAC-associated hexokinase, peripheral benzodiazepine receptor [6], pro-apoptotic and anti-apoptotic Bcl-2 family proteins could play a regulatory role.

Opening of MPTP is triggered by calcium overload. Several factors such as oxidative stress, adenine nucleotide depletion, increased inorganic phosphate and mitochondrial depolarization greatly enhance the sensitivity of the pore to Ca^{2+}. MPTP opening can be inhibited by cyclosporin A. Various peroxides such as *tert*-butylhydroperoxide, which are used as a model of oxidantive damage [22, 28, 39], can bring about the MPTP opening [17].

MPTP was discovered and studied on isolated mitochondria and mitochondrial swelling. However, some data have recently appeared indicating that mitochondrial contact with the endoplasmic reticulum and other structures as well as with other mitochondria are important for mitochondrial functional activity [15, 37]. Permeabilized cells by digitonin can be used as a useful biological model because the intracellular network is maintained [14] and internalized mitochondria are accessible for exogenous substrates and cofactors.

As TPP^+-selective electrode is very useful method for real-time $\Delta\psi_m$ monitoring, we constructed the computerized device using these electrodes and used this method for evaluation of $\Delta\psi_m$ and respiratory chain function in isolated mitochondria and mitochondria of isolated hepatocytes and cultured cells permeabilized by digitonin. 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.

Because peroxides facilitate MPTP opening, we used the TPP^+-selective electrodes for assessing the MPTP function under oxidative stress and enhancing the effect of calcium on MPTP opening.

2. AIM OF THE STUDY

The goal of this study was to construct the computerized device for membrane potential measurement using the TPP^+-selective electrode, optimize TPP^+-selective membrane composition and calculate selectivity coefficients of the optimized membrane for various ions. The second goal was to test the constructed device by measuring $\Delta\psi_m$ of isolated mitochondria and to evaluate $\Delta\psi_m$ in digitonin-permeabilized cells, where $\Delta\psi_m$ is assessed *in situ*.

1. The construction of the device for membrane potential measurement using TPP^+-selective electrode

- The construction of the apparatus for membrane potential measurement using TPP^+-selective electrode
- The connection to the computer and data acquisition, filtration and storage in MATLAB/Simulink software
- Optimization of the TPP^+-selective membrane
- Calculation of the selectivity coefficients of the optimized electrode for various ions

2. Measurement of the mitochondrial membrane potential using the constructed device

- Measurement of the mitochondrial membrane potential of isolated mitochondria – evaluation of the respiratory chain function.
- Measurement of $\Delta\psi_m$ *in situ* - $\Delta\psi_m$ of isolated hepatocytes and cultured cells permeabilized by digitonin. Evaluation of their respiratory chain function.
- The action of calcium and pro-oxidant *tert*-butylhydroperoxide on $\Delta\psi_m$ of isolated hepatocytes and isolated mitochondria. The involvement of the mitochondrial permeability transition pore.

3. RESULTS AND DISCUSSION

3.1 Construction of the device for membrane potential measurement

The computerized device for membrane potential measurement was constructed in our laboratory. It contains the measuring chamber (maximum sample volume of 5 ml) with the magnetic stirrer, the reference electrode, the TPP^+-selective electrode and PC which includes the high-impedance measuring card PCI-603E (National Instruments, USA) (Fig.1). Real-time signal acquisition, processing, display and data storage were realized by MATLAB/Simulink software (The MathWorks, Inc., USA). Fig. 2 shows the connection of the Simulink modules. The constructed device was placed into the Faraday cage to shield against electromagnetic radiation.

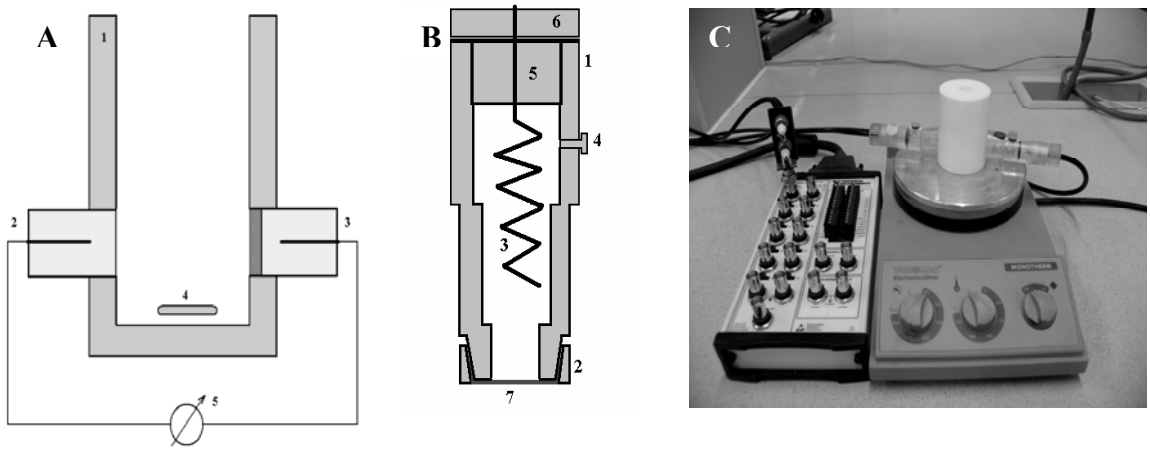

Fig. 1 **A** *Schematic diagram of the membrane potential measuring circuit: 1, the measuring chamber; 2, the reference Ag/AgCl electrode; 3, the TPP^+-selective electrode; 4, the magnetic stirrer; 5 PC with PCI-603E measuring card.* ***B*** *Construction details of the TPP^+-selective electrode: 1, the electrode body; 2, the Plexiglas ring; 3, the Ag/AgCl wire; 4, the opening for inner electrode 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 the electrodes assembly.*

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. 1 B. 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.

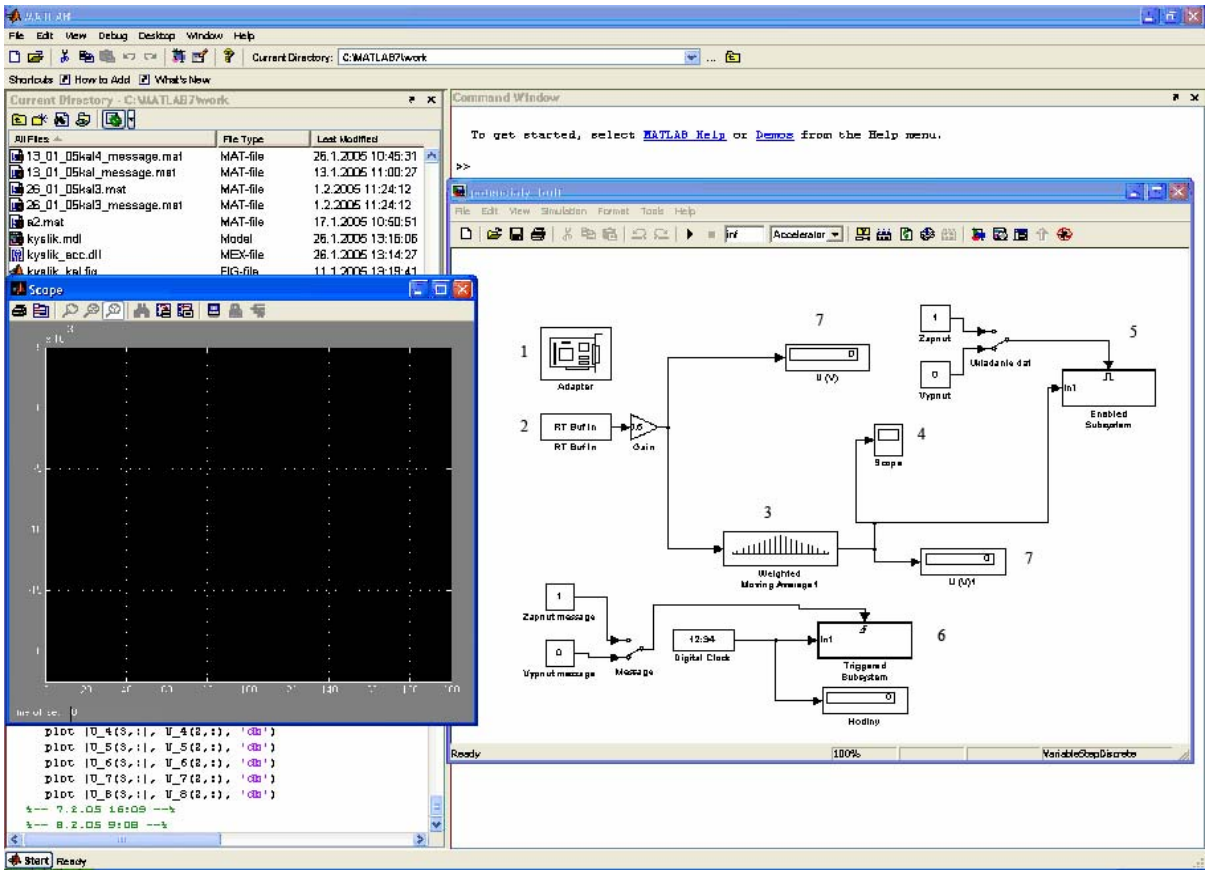

Fig. 2 *The Simulink modules connection.1, Adapter executes the communication with the measurement card ; 2, RT BufIn allows the adjustment of the buffer capacity and input parameters – frequency of the data acquisition and the input channel. The buffer was set to 1000 samples; 3, Data filtration realized by moving average filter; 4, Real-time display of the acquired data; 5, Data storage; 6, Recording of the user's message; 7 Data display.*

3.1.1 Preparation of the TPP^+-selective membrane

The PVC-based TPP^+-selective membranes were prepared according to Kamo et al. (1979) [21], which contained tetraphenyl boron (TPB^-). Three different types of PVC made in the Czech Republic were tested: Neralit 581, Neralit 682 and Naralit 702 (Spolana, Neratovice). The most suitable was Neralit 702 because of its elasticity and solidity.

We compared the response of the electrodes, which were filled with either 10 mM TPP^+Cl^- or with 10 mM TPP^+Br^- as the inner filling solution. The electrode filled with TPP^+Cl^- had a non-Nernstian 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.

(1978) [36]. Instead of Na^+TPB^- we added the precipitate of TPP^+Cl^- and Na^+TPB^- to the membrane. The modified TPP^+-selective electrode had the Nernstian response to TPP^+ from $3x10^{-6}$ M, which was independent of the filling solution (10 mM TPP^+Cl^- or TPP^+Br^-). The electrode with the new prepared membrane had a stable and reproducible response for 2 to 3 months. The response time of the electrode was 4 – 6 s.

Because TPP^+ was washed out from the modified membrane, we replaced the precipitate by sodium tetrakis[3,5-bis(1,1,1,3,3,3-hexafluoro-2-methoxy-2-propyl)phenyl]borate (Na^+HFPB^-) which is more stable than Na^+TPB^- [4]. TPP^+Cl^- (10 mM) was used as the inner filling solution. The prepared electrode had a reproducible Nernstian response from $1x10^{-6}$ M (Fig. 3) and the response time was 8-12 s due to the ion-exchange at the membrane/sample interface.

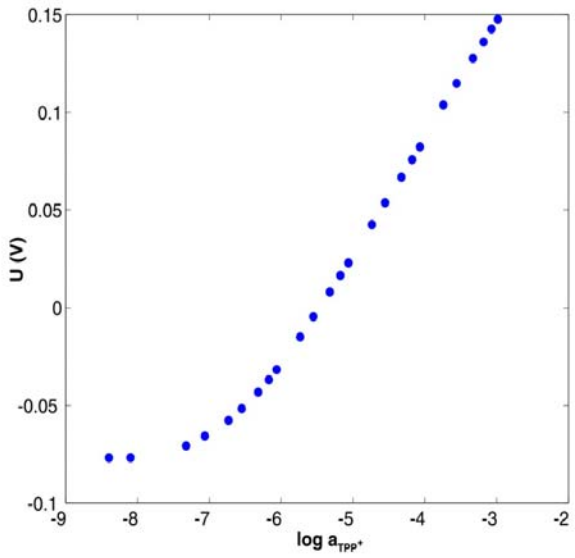

Fig. 3 *Calibration curve of the TPP^+-selective electrode with the membrane containing $HFPB^-Na^+$. The calibration was done in 0.1 M NaCl background.*

In contrast with our improved electrodes, commercially available TPP^+-selective electrodes (World Precision Instruments) have the Nernstian response from $10^{-4,5}$ M TPP^+. Therefore, our electrodes are more suitable for membrane potential measurement due to their higher sensitivity in the concentration range 10^{-5} – 10^{-6} M TPP^+ where the membrane potential is usually measured.

3.3.2 Calculation of the selectivity coefficients of TPP^+-selective electrodes

The selectivity coefficients (K_{ij}^{pot}) for the TPP^+-selective membrane with the incorporated TPP^+TPB^- were calculated according to the *Fixed interference method* for K^+ ((4.23±0.97)x10^{-6}) and Na^+ ((4.38±2.29)x10^{-6}) and from the Nicolskii-Eisenman equation (the nonlinear method of least squares) ((5.126±0.70)x10^{-6} for K^+) and (3.40±1.06)x10^{-6} for Na^+). The calculated selectivity coefficients were influenced by TPP^+ washing out from the membrane. Therefore, their values could be overestimated by this process.

The selectivity coefficients were calculated (see Tab. 1) also for the TPP^+-selective membrane with incorporated Na^+HFPB^-, where TPP^+ washing out was minimized.

j	K^+	Na^+	Ca^{2+}	Mg^{2+}
$K_{TPP,j}^{pot}$ (IUPAC)	(2.31±0.34)x10^{-6}	(1.94±0.56)x10^{-6}	(4.76±0.68)x10^{-7}	(5.29±1.60)x10^{-7}
$K_{TPP,j}^{pot}$ (NE)	(2.48±0.58)x10^{-6}	(1.73±0.13)x10^{-6}	(5.01±0.55)x10^{-7}	(5.78±0.22)x10^{-7}
$K_{TPP,j}^{pot}$ (Bakker et al.)	-	-	(5.46±0.56)x10^{-7}	(7.1±2.5)x10^{-7}

Tab.1 *The values of selectivity coefficients calculated according to the IUPAC, from the Nicolskii-Eisenman equation (NE) (3) and equation according to Bakker et al. (5). (Electrode with incorporated Na^+HFPB^-)*

Except the Nicolskii-Eisenman equation (3) and the equation according to Bakker et al. (5), the equation for small interference (6) was used. We found that this equation for K_{ij}^{pot} calculation is not convenient (see Fig. 4). The Nicolskii-Eisenman equation (for univalent and bivalent cations) and the equation according to Bakker et al. (5) (for bivalent cations) are both suitable, but the Nicolskii-Eisenman equation is easier to compute.

The only selectivity coefficient for K^+ ($K_{TPP,K}^{pot} = 10^{-6}$) of the electrode made by World Precision Instruments according to the World Precision Instruments catalogue [9] was $K_{TPP,K}^{pot} = 10^{-6}$. $K_{TPP,K}^{pot}$ of this electrode is comparable with the calculated value of $K_{TPP,K}^{pot}$ (Tab.1) of our improved electrode with incorporated Na^+HFPB^-.

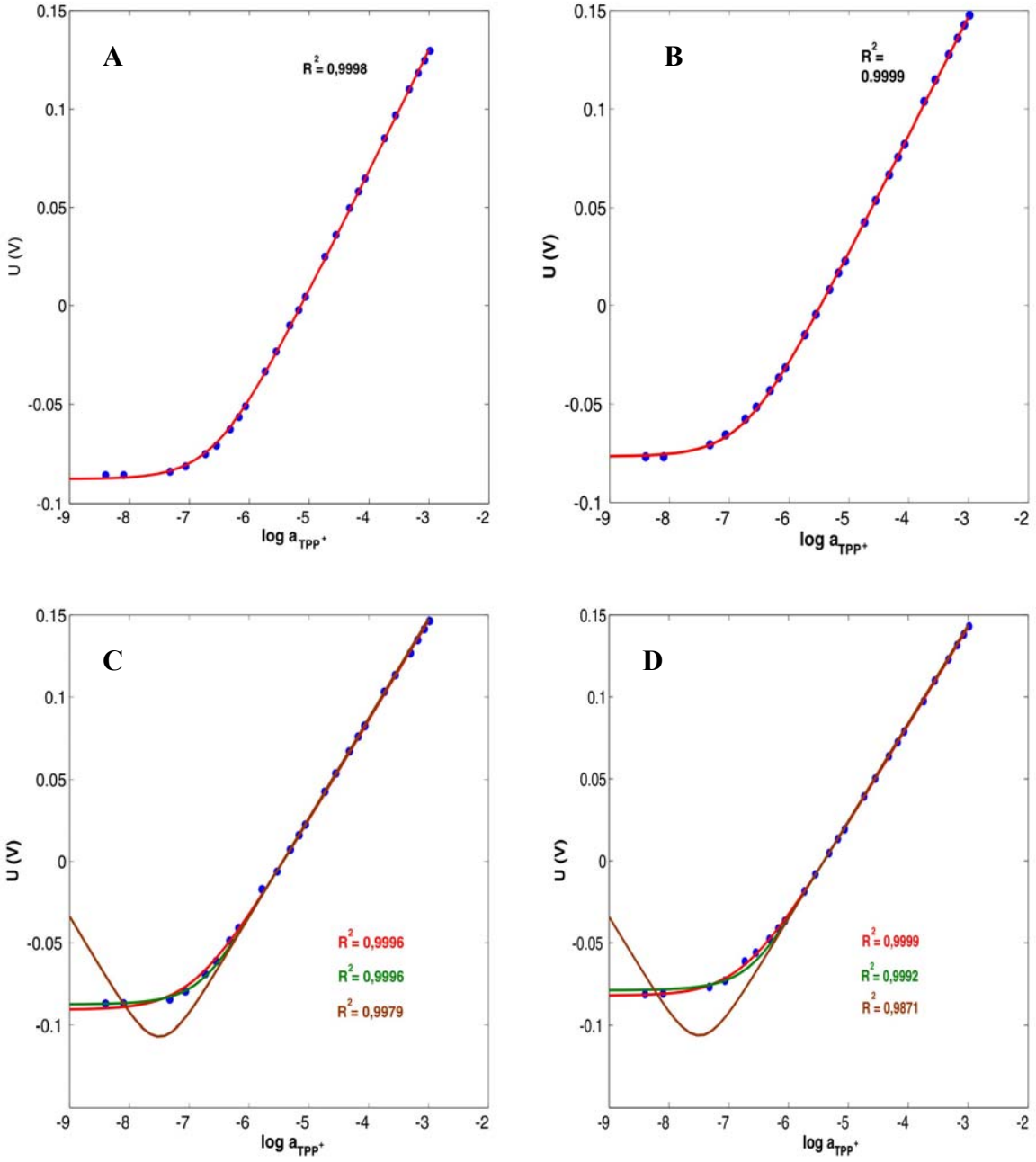

Fig.4 *Calibration curve of the TPP^+-selective electrode with incorporated Na^+HFPB^- in the* ***A*** *K^+(0.1 M KCl) background,* ***B*** *Na^+ background (0.1 M NaCl),* ***C*** *Mg^{2+} background and* ***D*** *Ca^{2+} background and the model curves according to the Nicolskii-Eisenman equation (-), Bakker et al. (1997)(5) and (-),equation for small interference (6) (-).*

3.2 Measurement of the mitochondrial membrane potential

3.2.1 Mitochondrial membrane potential of isolated rat mitochondria and evaluation of their respiratory chain function

The constructed device was used for evaluation of the mitochondrial membrane potential of isolated mitochondria. To quantify the amount of TPP^+ accumulated in mitochondia, the TPP^+-electrode was calibrated by successive addition of TPP^+ before each measurement (Fig. 5 A). Changes in mitochondrial membrane potential due to the addition of the substrates and inhibitors of the respiratory chain are presented as changes of TPP^+ concentration (Fig. 5 B).

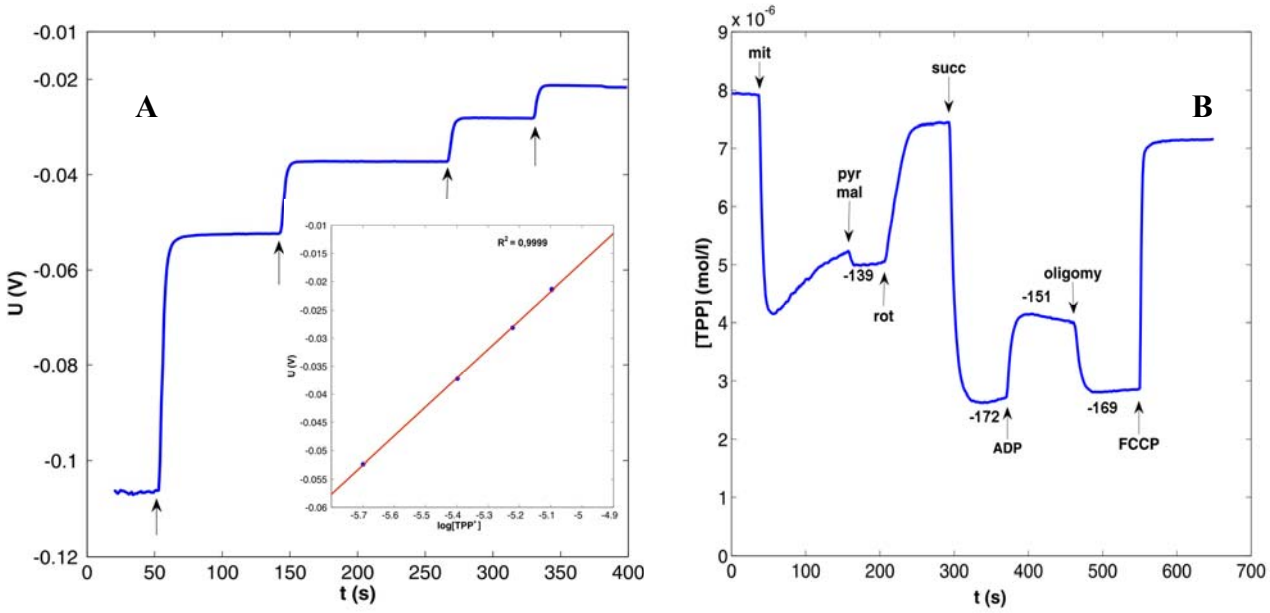

Fig. 5 A *Calibration of TPP^+-selective electrode in concentration range 2 – 8 μM TPP^+. Arrows indicate addition of TPP^+ to final concentration: 2μM, 4 μM, 6μM, 8μM. (Inset) Electrode response as a function of logarithm of TPP^+ concentration.* ***B*** *Analysis of $\Delta\psi_m$ of isolated rat mitochondria. Arrows indicate the addition of mitochondria (mit, 0.3 mg/ml), pyruvate (pyr, 10 mM), malate (mal 2.5 mM), rotenone (rot, 1 μM), succinate (succ, 10 mM), ADP (0.5 mM), oligomycin (oligomy, 1 μM), FCCP (1 μM).*

The analysis of the changes in $\Delta\psi_m$ of isolated rat liver mitochondria was performed in 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). After the 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 (the substrates of NADH-dependent dehydrogenases) prevented the TPP^+ concentration 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. ADP was added to test the function of ATP synthase, which caused the release of TPP^+ indicating a partial depolarization. Oligomycin, a specific inhibitor of ATP synthase, restored $\Delta\psi_m$ and the uncoupler FCCP decreased $\Delta\psi_m$ to a minimum value. The $\Delta\psi_m$ dissipation was caused also by the addition of antimycin A and myxothiazol – specific inhibitors of complex III (not shown).

The value of $\Delta\psi_m$ included in Fig. 5 was calculated using the equation:

$$\Delta\psi_m = \frac{RT}{F}\ln\frac{V_0[TPP^+]_0/[TPP^+]_t - V_t - K_0P}{V_mP + K_iP}, \qquad (9)$$

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), $[TPP^+]_0$ and $[TPP^+]_t$ are the concentrations of TPP^+ prior to the 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 coefficient of TPP^+ [40]. In parallel experiments on the oxygraph we confirmed that mitochondria used for measurements were tightly coupled, with a respiratory control index of 4-6.

The obtained data are in accordance with the previously published studies [21, 26, 29].

3.2.2 Mitochondrial membrane potential of isolated rat hepatocytes and cultured cells. Evaluation of their respiratory chain function

The mitochondrial membrane potential was analyzed in isolated rat hepatocytes and cultured cells (HeLa G, BSC-40 and control transmitochondrial cybrids) permeabilized by small amount of digitonin. Fig. 6 A shows TPP^+ concentration change upon their permeabilization with digitonin and subsequent addition of substrates and inhibitors of the respiratory chain. Measurements were performed in the K-medium. After the addition of hepatocytes, small accumulation of TPP^+ occurred. Addition of digitonin caused collapse of the plasma membrane potential and pronounced decrease of TPP^+ concentration in the

medium due to the probe accumulation in the mitochondria. The changes in $\Delta\psi_m$ after subsequent addition of substrates and inhibitors were similar to $\Delta\psi_m$ changes of isolated mitochondria. Parallel experiments on the oxygraph showed that under similar conditions the mitochondria in permeabilized hepatocytes were coupled and the changes after addition of substrates and inhibitors corresponded to the measurements of $\Delta\psi_m$ [23]. The value of $\Delta\psi_m$ = 0 is represented by TPP^+ concentration after FCCP addition (Fig. 8 B).

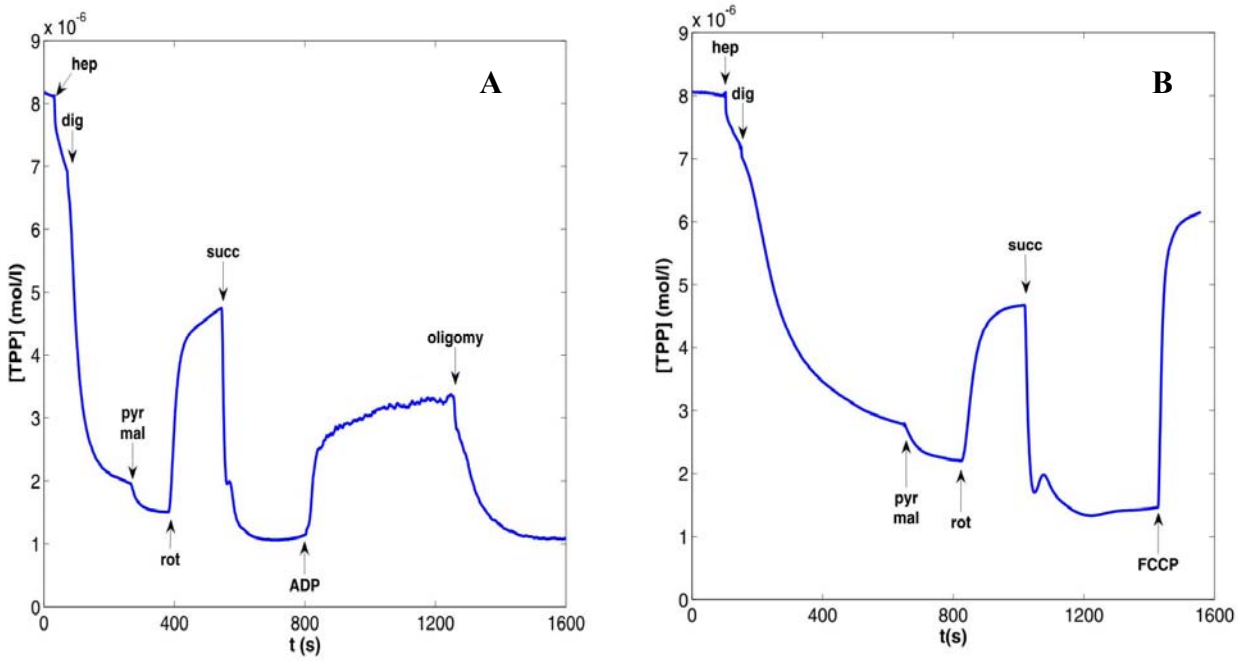

Fig. 6 *Analysis of $\Delta\psi_m$ of isolated hepatocytes. Arrows indicate the addition of* **A** *hepatocytes (hep, 1.65 million cells/ml), digitonin (dig, 0.035 mg/ml), pyruvate and malate,(mal, 2.5 mM; pyr,10 mM), rotenone (rot, 1 μM), succinate (succ, 10 mM), ADP (0.5 mM), oligomycin (oligomy, 1 μM);* **B** *hepatocytes (1.65 million cells/ml), digitonin (0.035 mg/ml), pyruvate and malate (10 mM, 2.5 mM) , succinate (10 mM), FCCP (1 μM).*

This approach was used also for assessing mitochondrial function in other types of cultured cells such as control transmitochondrial cybrids, HeLa G or BSC-40. The changes in $\Delta\psi_m$ after addition of substrates and inhibitors of the respiratory chain were similar to $\Delta\psi_m$ changes of isolated hepatocytes. The amount of the sample per ml of medium needed for good response was three to fivefold higher than the amount of hepatocytes. The optimal concentration of digitonin was tested for each cell type (HeLa G and BSC-40 – 0.075 mg/ml; cybrids – 0.1 mg/ml).

3.2.3 The evaluation of the mitochondrial permeability transition pore function

TPP^+-selective electrodes were also used for $\Delta\psi_m$ monitoring during the mitochondrial permeability transition pore (MPTP) opening in isolated rat mitochondria and permeabilized rat hepatocytes. MPTP opening and consequent $\Delta\psi_m$ dissipation was induced by high concentration of calcium (100 μM) (Fig.7) as was previously described [20, 27].

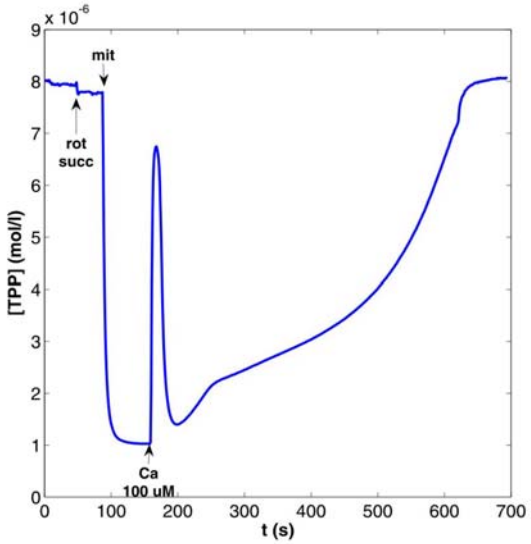

Fig. 7 *TPP^+ concentration changes after Ca^{2+}addition. Arrows indicate addition of rotenone (rot, 1 μM), succinate (succ, 10 mM), mitochondria (mit, 0.4 mg/ml) and Ca^{2+}(Ca, 100 μM).*

The process of the MPTP opening can be enhanced at low Ca^{2+} concentration by oxidative stress. In the presence of 1.5 mM pro-oxidant *tert*-butylhydroperoxide (*t*-BHP) and low calcium concentration (12.5 μM) the dissipation of $\Delta\psi_m$ occurred (Fig. 8 A). This dissipation was inhibited by cyclosporin A (Fig. B), which indicates that $\Delta\psi_m$ decrease was caused by MPTP opening. Low calcium concentration (12.5 μM) and *t*-BHP (1.5 mM) did not induce MPTP opening (Fig. 8 C and D).

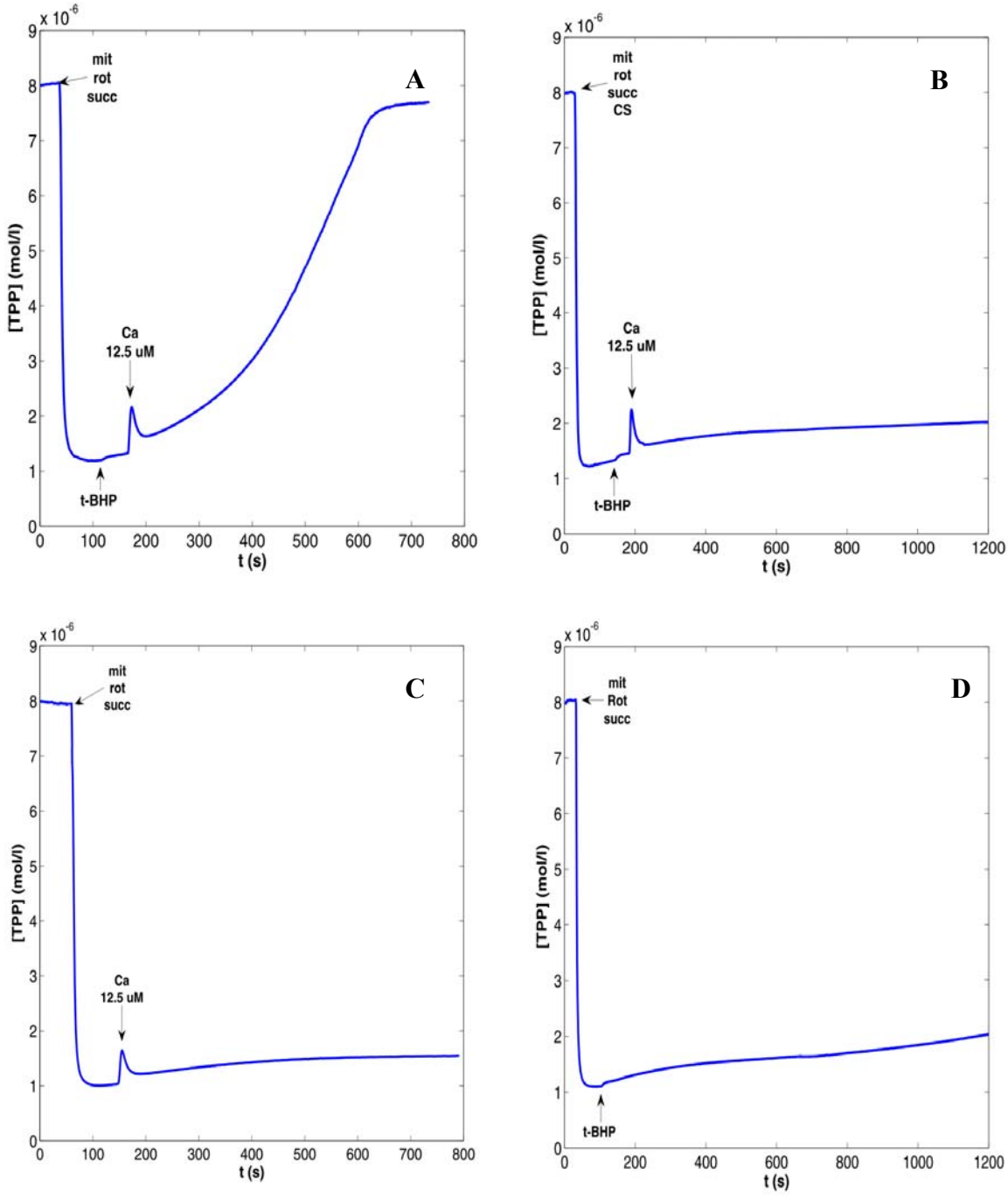

Fig. 8 *TPP^+ concentration changes due to calcium and t-BHP addition. Arrows indicate addition of mitochondria (mit, 0.4 mg/ml), rotenone (rot, 1 μM), succinate (succ, 10 mM), Ca^{2+} (Ca, 12.5 μM), t-BHP (1.5 mM) and cyclosporin A (CS, 2 μM).*

When we tested changes of the $\Delta\psi_m$ in hepatocytes permeabilized by digitonin and energized by succinate (in K-medium without EDTA which did not influence Ca^{2+} concentration in the medium), we found that the membrane potential is maintained only for a short period of time after digitonin addition (about 4 min.) and then quickly dissipated (Fig. 9 A). This dissipation was reversed by the addition of ethylene glycol-bis-(β-aminotethylether) tetraacetic acid (EGTA) or it was completely prevented by cyclosporin A (Fig.9B). This indicates involvement of Ca^{2+}-activated MPTP function. The effect was concentration-dependent and in agreement with previous findings [18] at higher Ca^{2+} concentration (above 250 μM), $\Delta\psi_m$ dissipated even in the presence of cyclosporin A (Fig. 9B).

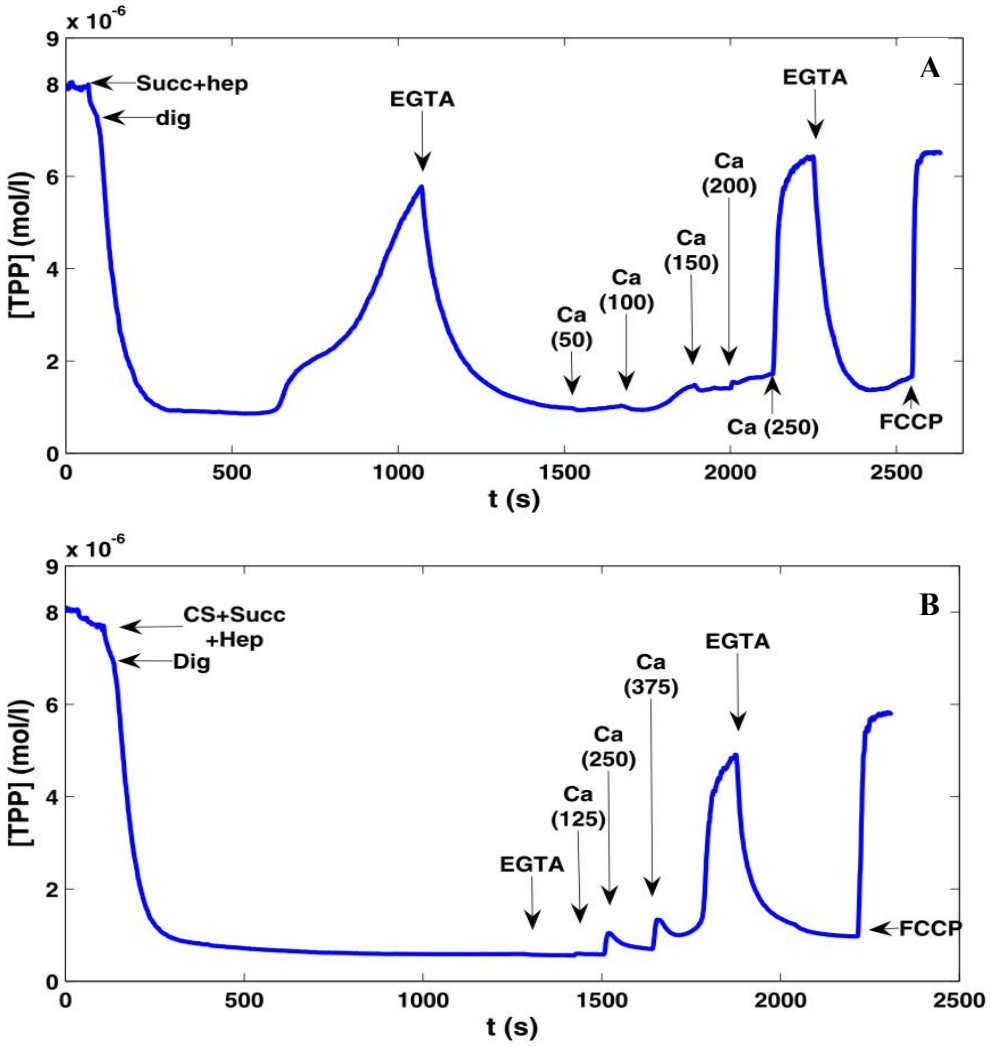

Fig. 9 *TPP^+ concentration changes induced by calcium and EGTA.. Arrows indicate addition of* ***A*** *succinate (succ, 10 mM), hepatocytes (hep, 1.64 million cells/ml), digitonin (dig, 0.035 mg/ml), EGTA (350 μM), Ca^{2+} (Ca, 50, 100, 150, 200 μM), EGTA (3 mM), FCCP (1 μM);* ***B*** *succinate (10 mM), hepatocytes (1.64 million cells/ml), cyclosporin A (CS, 2 μM), digitonin (0.035 mg/ml), EGTA (350 μM),Ca^{2+} (125, 250, 375 μM), EGTA (3 mM), FCCP (1 μM).*

These findings show that Ca^{2+} present in the medium during hepatocyte isolation cannot be completely removed by repeated washing, 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 effect of *t*-BHP, we used medium with 1 mM EDTA that completely prevented the dissipation of $\Delta\psi_m$ by contaminating Ca^{2+} ions (Fig. 10 A). Under these experimental conditions, the $\Delta\psi_m$ was not affected by Ca^{2+} added in concentrations below 200 μM (Fig. 10 A). Also *t*-BHP up to 1.5 mM in the medium with 1 mM EDTA had no dissipating effect (Fig. 10 B). However, when 1.5 mM *t*-BHP was added in the presence of 50 μM Ca^{2+}, the $\Delta\psi_m$ was dissipated (Fig. 10 C). The dissipation was prevented by cyclosporin A (Fig. 10 D). This confirms previous observations indicating that oxidative stress increases the sensitivity of MPTP to Ca^{2+} [18].

We also tested rotenone, a potent inhibitor of MPTP [19], but it did not completely inhibit $\Delta\psi_m$ dissipation compared to cyclosporin A (not shown).

3.2.4 t-BHP action on mitochondrial respiratory chain function

The action of *t*-BHP on mitochondrial respiratory chain function was studied in digitonin-permeabilized rat hepatocytes in K-medium. According to Drahota et al. (2005) [13] critical concentration for respiratory chain inhibition was 0.75-3 mM *t*-BHP. Therefore, we used the concentration range 0.3 – 3 mM *t*-BHP. The *t*-BHP action is time and concentration dependent.

We compared the $\Delta\psi_m$ changes in the presence of *t*-BHP and different substrates of the respiratory chain – pyruvate + malate and succinate. When pyruvate and malate were present in the medium, addition of *t*-BHP (0.5, 1.5 and 3 mM *t*-BHP) caused $\Delta\psi_m$ dissipation (Fig. 11 A). This process was not cyclosporin A-sensitive (Fig. 11 B). Mitochondria were again energized by the addition of succinate. In the case of mitochondria energized by succinate (complex I inhibited by rotenone), $\Delta\psi_m$ dissipated in the presence of 3 mM *t*-BHP (Fig. 11 C) due to the lower sensitivity of complex II to oxidative damage [13]. The $\Delta\psi_m$ dissipation was completely inhibited by cyclosporin A (Fig. 11 D).

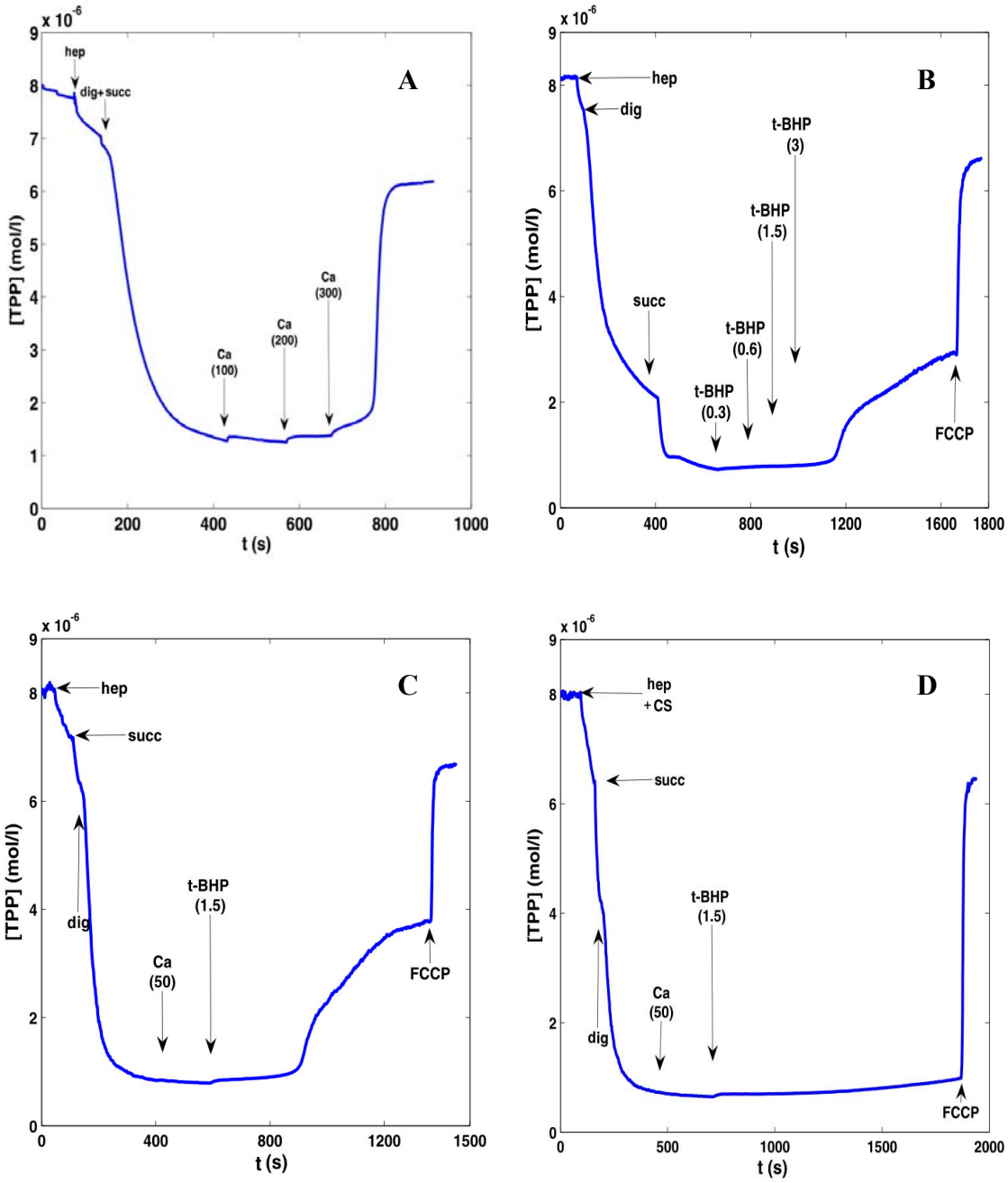

Fig. 10 *Changes of TPP^+ concentration in the medium induced by calcium and t-BHP. Hepatocytes were incubated in K-medium with 1 mM EDTA. Arrows indicate addition to the final concentration of* **A** *succinate (succ, 10 mM), hepatocytes (hep, 1.8 million cells/ml), digitonin (dig, 0.035 mg/ml), Ca^{2+} (Ca, 100 μM), Ca^{2+} (200 μM), Ca^{2+} (300 μM);* **B** *succinate (10 mM), hepatocytes (1.8 million cells/ml), digitonin (0.035 mg/ml), t-BHP (0.3 mM), t-BHP (0.4 mM), t-BHP (1.5 mM), t-BHP (3 mM), FCCP (1 μM);* **C** *succinate (succ, 10 mM), hepatocytes (1.64 million cells/ml), digitonin (0.035 mg/ml), Ca^{2+} (50 μmol/l), t-BHP (1.5 mM), FCCP (1 μM);* **D** *hepatocytes (1.64 million cells/ml), cyclosporin A (CS, 2 μM), succinate (10 mM), digitonin (0.035 mg/ml), Ca^{2+} (50 μM), t-BHP (1.5 mM), FCCP (1 μM).*

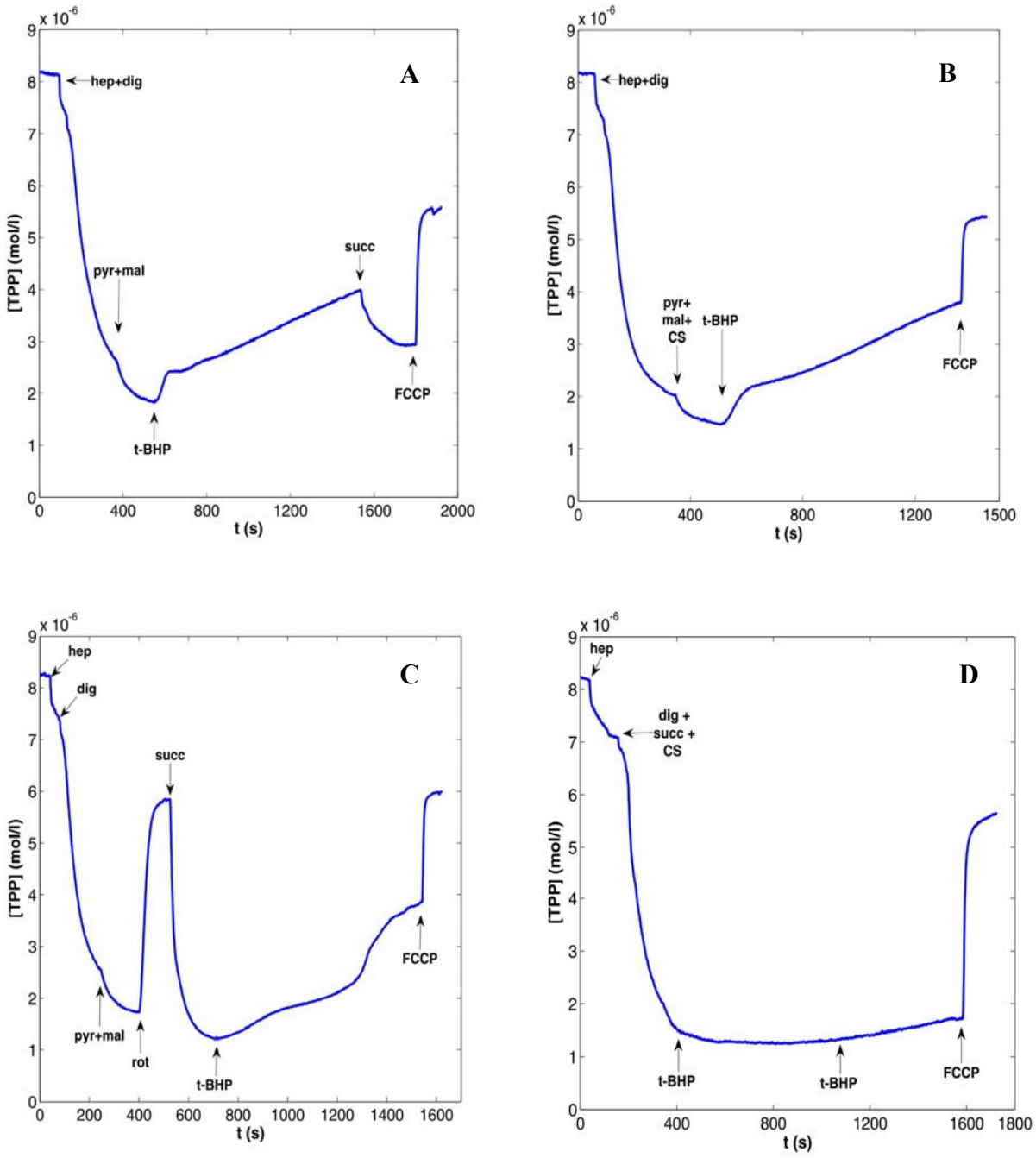

Fig. 11 *Changes of TPP^+ concentration in the medium induced by t-BHP. Arrows indicate addition of* ***A*** *hepatocytes (hep 1.8 million cells/ml), digitonin (dig, 0.035 mg/ml), pyruvate and malate (pyr, 10 mM, mal 2.5 mM) t-BHP (1.5 mM), succinate (succ, 10 mM), FCCP (1μM),* ***B*** *hepatocytes (1.8 million cells/ml), digitonin (0.035 mg/ml), pyruvate and malate (10 mM, 2.5 mM), cyclosporin A (CS, 2 μM), t-BHP (1.5 mM), FCCP (1μM),* ***C*** *hepatocytes (1.8 million cells/ml), digitonin (0.035 mg/ml), pyruvate and malate (10 mM, 2.5 mM), rotenone (rot, 1μM), succinate (10 mM), t-BHP(3 mM), FCCP (1μM),* ***D*** *hepatocytes (1.8 million cells/ml), digitonin (0.035 mg/ml), rotenone (1μM), succinate (10 mM), cyclosporin A (2 μM), t-BHP (1.5 and 3 mM), FCCP (1μM).*

Our findings confirm the uncoupling effect of *t*-BHP (by 50 %) measured by respiration rate of mitochondria [42].

The action of *t*-BHP on $\Delta\psi_m$ of isolated hepatocytes may be summarized as:

1. Dissipation of the $\Delta\psi_m$, which is not cyclosporin A-sensitive, is probably caused by inhibition of complex I due to modification of Fe-S centers of complex I by oxidative damage [13] or due to mitochondrial aconitase inhibition [8].
2. Uncoupling effect of *t*-BHP, which is cyclosporin A-sensitive, could be caused by opening of MPTP.

We may thus conclude that our constructed device for measuring the $\Delta\psi_m$ changes can be used for the evaluation of hepatotoxic action of various agents that damage hepatocyte energy metabolism due to the activation of MPTP opening and other factors that influence the $\Delta\psi_m$.

4. CONCLUSION

1. Construction of the device for membrane potential measurement

The computerized device for membrane potential measurement using the TPP^+-selective electrode was constructed. Data acquisition and subsequent data processing was realized by MATLAB/Simulink software.

The PVC-based sensitive membrane composition of the TPP^+-selective electrode was changed by the addition of TPP^+TPB^- precipitate. This electrode had a Nernstian response to TPP^+ from $3x10^{-6}$ M TPP^+. Because TPP^+ was washing out from the modified membrane, the precipitate was changed by sodium tetrakis[3,5-bis(1,1,1,3,3,3-hexafluoro-2-methoxy-2-propyl)phenyl]borate. This membrane had Nernstian response to TPP^+ from $1x10^{-6}$ M TPP^+ and TPP^+ did not wash out from the membrane to the medium. Therefore, it was possible to compute the selectivity coefficients for Na^+, K^+, Ca^{2+} and Mg^{2+} according to the IUPAC recommendation, the Nicolskii-Eisenman equation and the equation that respects the different valency of primary and interfering ions (5) (Bakker et al., 1997). In the case of univalent cations, the Nicolskii-Eisenman equation is suitable for describing the calibration of the electrode. For divalent cations, the proper model of the calibration curve is the Nicolskii-Eisenman equation and the equation according to Bakker et al. (1997), however the Nicolskii-Eisenman equation is easier to compute.

The constructed device is suitable for TPP^+ concentration measurement that allows real-time monitoring of the membrane potential. Optimization of the TPP^+-selective membrane decreased the detection limit compared to the commercially available electrode (World Precision Instruments). Thereby the sensitivity of the TPP^+ concentration measurement in the concentration range 10^{-5}-10^{-6} M increased. This concentration range is suitable for membrane potential measurement.

2. Measurement of the mitochondrial membrane potential

The constructed device was tested by measuring $\Delta\psi_m$ in isolated mitochondria. The TPP^+ concentration changes in the medium were dependent on the addition of various substrates and inhibitors of the respiratory chain. The measured data were in accordance with the data previously published.

Mitochondrial membrane potential was also measured in digitonin-permeabilized cells. In these experimental conditions, the mitochondrial membrane potential is measured *in situ* and the intracellular network is maintained. $\Delta\psi_m$ of the permeabilized hepatocytes, HeLa G, BSC-40 and control transmitochondrial cybrids was evaluated as changes of TPP^+ concentration in the medium.

This method was used for monitoring the mitochondrial permeability transition pore function in isolated mitochondria and mitochondria of permeabilized hepatocytes. MPTP was induced by high Ca^{2+} concentration. The action of Ca^{2+} at low concentration was enhanced by pro-oxidant *tert*-butylhydroperoxide. The process was inhibited by cyclosporin A. After plasma membrane permeabilization of isolated hepatocytes (in the medium without EDTA) MPTP opened due to the Ca^{2+} preload during the isolation procedure or due to the presence of Ca^{2+}, which could not be removed by repeated washing.

The action of the pro-oxidant *t*-BHP on respiratory chain function was studied in isolated hepatocytes. We propose that *t*-BHP dissipation was caused by the inhibition of the complex I or mitochondrial aconitase. *t*-BHP induced MPTP opening as well.

The $\Delta\psi_m$ measurement using TPP^+-selective electrodes in isolated mitochondria and permeabilized cells showed that this technique is useful for studies of many aspect of the mitochondrial bioenergetics. It may also be used as a tool for study of mitochondrial disorders and their diagnostics. In addition, the $\Delta\psi_m$ changes can be used for the evaluation of hepatotoxic action of various agents that damage energy metabolism due to the activation of MPTP opening, which is the first step in apoptotic and necrotic processes.

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