Evaluation of cell envelope barrier and energetic functions of eukaryotic microorganisms using the lipophilic cations
II. Resolution of two-compartment problem in eukaryotic cell: estimation of plasma membrane and mitochondrial inner membrane voltages in *Trypanosoma brucei*

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The magnitudes of plasma membrane and mitochondrial inner membrane voltages ($\Delta\psi_p$ and $\Delta\psi_m$, respectively) were estimated in the cells of the unicellular eukaryotic microorganism *Trypanosoma brucei* by the optimized potentiometric method based on the measurements of accumulation of lipophilic cation tetraphenylphosphonium (TPP^+) by the cells. Selective inhibition of one kind of membrane voltage enabled to estimate the other. $\Delta\psi_m$ value of the trypanosome was calculated to be –160 mV upon dissipation of $\Delta\psi_p$ by osmotic swelling or by treatment of cells at a low temperature. The $\Delta\psi_p$ value was calculated to be –115 mV upon oligomycin-facilitated inhibition of $\Delta\psi_m$ generation.

Key words: *Trypanosoma brucei*, tetraphenylphosphonium, membrane voltages

INTRODUCTION

Membrane voltages ($\Delta\psi$) play an important role in the overall energy metabolism of the microbial cell [1]. The understanding of vital cellular functions requires estimation of exact voltage values. Cells of prokaryotic microorganisms comprise the one-compartment membranous systems with the single membrane voltage generated on the cellular plasma membrane. However, most eukaryotic cells maintain additional to plasma membrane voltages across the membranes of intracellular organelles (*e.g.*, mitochondria). Therefore the multicompartment problem should be solved to estimate the separate contributions of different $\Delta\psi$ to the accumulation of voltage-sensitive probes in the whole cell [1, 2]. In the present study, plasma membrane voltage ($\Delta\psi_p$) and mitochondrial inner membrane voltage ($\Delta\psi_m$) were assayed in the unicellular parasite *Trypanosoma brucei*. The existence of $\Delta\psi_p$ in bloodstream forms of *T. brucei* was first detected only in 1983, and it was believed to be a single membrane voltage of these parasitic cells for the decade [3, 4]. However, subsequent studies have demonstrated the presence of additional intracellular membrane voltage, and the single mitochondrion of *T. brucei* was identified to be this energized compartment [5, 6]. There is no evidences for the existence of endosomal or glycosomal membrane voltages in *T. brucei*. Intensive investigations of membrane voltages in *T. brucei* have begun only recently [2, 5, 6, 7, 8, 9]. Yet, different experimental conditions were used by all researchers, and there is a reason to believe that reliable values of membrane voltages probably have never been established in truly physiological conditions. The first aim of this work was to distinguish between membrane voltages ($\Delta\psi_p$ and $\Delta\psi_m$) in *T. brucei* bloodstream form. The second goal was to estimate the magnitudes of voltages in nearly identical to physiological trypanosome conditions by using an optimized potentiometric method described in our previous study (10).

MATERIALS AND METHODS

Reagents. Tetraphenylphosphonium chloride was purchased from Aldrich, gramicidin D, oligomycin, sodium citrate were purchased from Sigma, D-glucose was purchased from Roth, all other reagents were of analytical grade.

Trypanosomes. *Trypanosoma brucei brucei* strain WM 14 221 (VSG 221) was kindly provided by Prof. James Bangs (University of Wisconsin, Madison, USA).

General methods. Growth, isolation and counting of bloodstream forms of *T. brucei* were performed as described previously [11]. Isolation and subsequent maintenance (for up to 1.5 h) of the concentrated cell suspension (5×10^9 cells/ml) before measurements were carried out at room temperature (unless specified otherwise) in order to prevent disturbance of their $\Delta\psi_p$ [7]. Trypanosome incubation medium contained 5 mM KCl, 80 mM NaCl, 1 mM $MgSO_4$, 20 mM $Na_2PO_4 \times 2H_2O$, 20 mM glucose, pH 7.4. The low-osmotic-strenght buffer (100 mOsM) contained 7 mM KCl, 30 mM NaCl, 0.5 mM $MgSO_4$, 5 mM Na_2HPO_4, 5 mM Hepes, pH 7.3. Cell counts were performed using an SK14 PZO microscope (Poland).

Measurements of TPP^+ distribution were performed by using selective electrodes as described earlier [12]. $\Delta\psi$ was calculated from the modified Nernst equation:

$$\Delta\psi_{(p/m)} = -RT/F \ln\{V_o/V_i[(C_b/C_a)^{\Delta b-\Delta c/\Delta a}-1]\},$$

where V_o is the volume of incubation medium, V_i is the the volume of internal water of the compartment (cells or mitochondria) [5]; C_a and C_b are concentations of TPP^+ before and after calibration, respectively; Δa is the change in the potential of the electrode during calibration; Δb is the change in the potential of the electrode due to the TPP^+ uptake by the cells; Δc is the change in the potential of the electrode due to the voltage-independent binding of TPP^+ by the cells.

RESULTS AND DISCUSSION

In the present study, to distinguish between *Trypanosoma brucei brucei* membrane voltages, we used the optimized potentiometric method based on the measurements of TPP^+ accumulation by the cells.

Two membranes of bloodstream forms, the plasma membrane and the mitochondrial membrane, are proved to have an electrochemical proton gradient across them. However, the first study on this subject demonstrated existence of only $\Delta\psi_p$ [3, 4]. This observation was consistent with the previous view in the literature, where the mitochondrion of the bloodstream form of *T. brucei* has been assumed to be a rather inert structure. It was already known at that time that *T. brucei* had to adapt their metabolism to the passage from insect to mammal host. Particularly, the mitochondrial structure and functions in response to environmental conditions were altered during this transformation [13]. The insect form has functional mitochondria. In the long slender (LS) *T. brucei* form, which is prevalent during early stages of a mammalian bloodstream infection, the single mitochondrion (usually referred to as promitochondrion) is reduced to a peripheral canal with almost no cristae. The Krebs cycle is nonfunctional and cytochromes are absent [14]. LS mitochondria respire at a very high rate because of a unique terminal oxidase system [15]. However, this truncated respiratory chain does not generate a gradient of H^+, and oxidative phosphorylation does not occur. Thus, at this stage of their life cycle parasitic cells rely exclusively on glycolysis for ATP production [13]. It was shown that although repression of mitochondrial function is extensive in bloodstream forms, these cells possess an active mitochondrial F_1F_0 ATPase complex [16]. In 1991–1992 Nolan and Voorheis demonstrated the presence of at least two distinct membrane voltages in the bloodstream form of *T. brucei*: a voltage across the plasma membrane ($\Delta\psi_p$) and a mitochondrial inner membrane voltage ($\Delta\psi_m$). The latter is generated and maintained exclusively by the electrogenic translocation of H^+ catalysed by the oligomycin-sensitive F_1F_0-ATPase, which operates in the direction of ATP hydrolysis without any contribution from the abbreviated electron transport chain [2, 5]. Selectively abolishing either the $\Delta\psi_p$, or the $\Delta\psi_m$, it is possible to measure the magnitude of both of these voltages in intact cells. It is most expedient to estimate $\Delta\psi_m$ *in situ*, since attempts to isolate mitochondria of *T. brucei* usually result in fragmentation and damage of mitochondrial membranes [5].

In experiments depicted in Fig. 1, the $\Delta\psi_m$ was estimated after solving the two-compartment problem for the uptake of TPP^+. The plasma membrane of *T. brucei* cells was selectively permeabilized and the ψ_p abolished by swelling the cells in a buffer of low osmotic strength (100 mOsM). The degree of swelling was monitored using a microscope. It was reported previously that a careful swelling of trypanosomes by decreasing the osmotic strength of the suspending medium under controlled conditions was a mild and effective procedure for selectively permeabilizing the plasma membrane of *T. brucei*, which becomes freely permeable to small inorganic ions [2]. The mitochondrion remains intact under such conditions, because the osmolarity in the cytoplasm remains higher than in the extracellular medium due to the presence of intracellular proteins and other

charged macromolecules that cannot diffuse out of the cell [2]. Our control experiments confirmed that the rate of oxygen consumption by the mitochondrion in the swollen cells remained unaffected (data not shown). It can be seen (Fig. 1, curve 2) that osmotic stress caused a substantial decrease in the accumulation of TPP^+ by trypanosomes, compared with the ones in iso-osmolar conditions (Fig. 1, curve 1). Another way to collapse $\Delta\psi_p$ selectively was to use trypanosomes isolated at 4 °C and then kept on ice (Fig. 1, curve 3). Though this traditional technique of trypanosome isolation and storage is supposed to do no harm to the cells, it was shown elsewhere that low temperature treatments induced irreversible depolarization of trypanosomal plasma membrane [7]. In the single recently reported study on this subject matter the authors used cultured cells grown axenically [7]. Our results using trypanosomes grown in rat provide support to this presumption and help to explain the noticeable differences among the $\Delta\psi_p$ values estimated in various laboratories for the bloodstream form *T. brucei* cells [2, 5, 6, 7, 8, 9]. The selective collapse of $\Delta\psi_p$ enabled to distinguish the remaining membrane voltage, $\Delta\psi_m$, which was shown to have a value of approximately –160 mV, close to the value of –150 mV reported by other authors [5, 6]. Addition of the uncoupler CCCP resulted in dissipation of $\Delta\psi_m$ (Fig. 1, curves 2 and 3).

Selective depolarization of mitochondrial membrane was accomplished by inhibition of mitochondrial F_1F_0-ATPase by oligomycin (Fig. 1, curve 4). The evidence was given previously that there was no detectable oligomycin-sensitive ATPase activity present in plasma membranes purified from *T. brucei* cells [17]. Thus, the effect of oligomycin on the accumulation of TTP^+ by trypanosomes can be ascribed to the collapse of $\Delta\psi_m$ as the result of specific inhibition of the mitochondrial ATPase but not to the inhibition of an electrogenic pump in the plasma membrane. Gramicidin D was used to depolarize the cells completely (Fig. 1, curve 4) in order to estimate the voltage-independent binding of TTP^+ by the cells. Results shown in Fig. 1, curve 4, allowed to estimate $\Delta\psi_p$, which was calculated to be –115 mV. Comparison with the values obtained by other authors (from –40 mV to –160 mV) is irrelevant because of the different temperatures of incubation and other conditions chosen by different authors [3, 4, 6, 8, 9]. The mechanism of $\Delta\psi_p$ generation still remains under debate: some authors suggest the main role of an ATP-dependent electrogenic pump in this process [7]; according to the others, $\Delta\psi_p$ is determined almost completely by the potassium diffusion potential [6]. Obviously, further work invoking specific inhibitory analysis is necessary to provide a better understanding of the nature of this phenomenon.

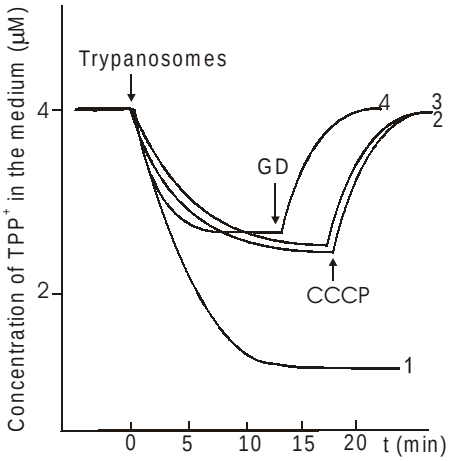

Fig. 1. Effects of the osmolarity of suspending medium, low temperature treatment and oligomycin on TPP^+ fluxes in *Trypanosoma brucei brucei* cells. The measurements were performed at 37 °C in trypanosome incubation medium (curves 1, 3 and 4) or low-osmotic-strenght buffer (curve 2). *Trypanosoma brucei brucei* WM 14 221 (VSG 221) cells isolated and kept room temperature (curves 1, 2 and 4) or at 4 °C (curve 3) were added to the final concentration of 2×10^8 cells/ml. Gramicidin D (GD) was added to the final concentration of 1 μg/ml, uncoupler carbonylcianide p-chlorophenylhydrazone (CCCP) – to the final concentration of 2 μM. In the experiment depicted in curve 4, oligomycin (final concentration 2 μg/ml) was present in the incubation medium before addition of the cells

In this study we provide the evidence for the usefulness and accuracy of the potentiometric method for the estimation of the membrane voltages of the eukaryotic microorganism *Trypanosoma brucei brucei.* It helps a broader and detailed understanding of the functions and properties of biological membranes of various origin and could have further implications in antiparasitic drug design.

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EUKARIOTINIŲ MIKROORGANIZMŲ APVALKALĖLIŲ BARJERINIŲ IR ENERGETINIŲ FUNKCIJŲ ĮVERTINIMAS LIPOFILINIŲ KATIJONŲ PAGALBA. II. DVIEJŲ KOMPARTMENTŲ PROBLEMOS EUKARIOTINĖJE LĄSTELĖJE SPRENDIMAS: *TRYPANOSOMA BRUCEI* PLAZMINĖS MEMBRANOS IR MITOCHONDRIJŲ VIDINĖS MEMBRANOS ĮTAMPŲ ATSKYRIMAS IR ĮVERTINIMAS

S a n t r a u k a

Potenciometriniu metodu, pagrįstu lipofilinių katijonų TPP^+ kaupimo ląstelėse matavimais, įvertintas eukariotinių mikroorganizmų *Trypanosoma brucei* membranos įtampų dydis. Selektyviai slopinant vieną iš membranų įtampų – plazminės membranos ar mitochondrijų vidinės membranos ($\Delta\psi_p$ ar $\Delta\psi_m$, atitinkamai), kiekybiškai nustatyta kitos įtampos skaitinė reikšmė. Tripanosomų $\Delta\psi_m$ dydis, apskaičiuotas nuslopinus $\Delta\psi_p$ generavimą ląsteles šaldant ar pažeidus plazmos membranos barjerą mažo osmosinio slėgio terpėje, buvo lygus –160 mV; $\Delta\psi_p$ reikšmė, apskaičiuota nuslopinus $\Delta\psi_m$ generavimą mitochondrijų ATPazės inhibitoriumi oligomicinu, buvo lygi –115 mV.