Evaluation of cell envelope barrier and energetic functions of eukaryotic microorganisms using lipophilic cations
I. Application of the potentiometric method for estimation of membrane voltages of *Trypanosoma brucei*

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We developed a potentiometric method for voltage estimation of *Trypanosoma brucei* cell membranes. The method is based on the measurements of accumulation of lipophilic cation tetraphenylphosphonium (TPP^+). Our work demonstrates that TPP^+ does not depolarize the cells up to concentrations of 2×10^{-5} M and therefore can be employed for potentiometric measurements. Both uptake of TPP^+ and oxygen consumption by *T. brucei* cells were slower at 24 °C as compared to the ones obtained at 37 °C. The steady-state in the distribution of TPP^+ was reached within about 10 min at 37 °C but only within about 35 min at 24 °C.

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

INTRODUCTION

Membrane-related processes are essential in energy metabolism of every cell. Membrane voltages (transmembrane differences of electrical potential, $\Delta\psi$) are among the main indicators of energetic state of the cell [1]. Studies with distribution of fluorescent probes or lipophilic ions are widely used for quantification of $\Delta\psi$ and frequently have contributed to our understanding of the role of $\Delta\psi$ in many important cellular processes, such as transport, electrical excitability, receptor-mediated signaling, etc. [1–4]. We developed a potentiometric method for measuring membrane voltages in the unicellular parasite *Trypanosoma brucei*, a commonly used eukaryotic cell laboratory model. Trypanosomes are protozoa of high medical and economic importance [5]. *Trypanosoma brucei* is an extracellular parasite that cycles between the bloodstream of mammalian host and digestive tract of the insect vector, the tse-tse fly (Glossina spp.). In sub-Sacharan and equatorial Africa two human infective subspecies, *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense*, are the causative agents of a grave human disease known as sleeping sickness. In addition, a third subspecies, which is not infective to humans, *Trypanosoma brucei brucei*, causes a fatal nagana disease in domestic livestock of the area [5, 6]. Within the past several years, these parasitic protozoa have drawn much attention, because they combine a number of unique properties, such as: 1) antigenic variation of surface proteins, 2) abundant occurrence of glycosylphosphatidyl-inositol (GPI)-anchored membrane proteins and glycolipids, 3) polycistronic transcription followed by trans-splicing of precursor mRNAs, 4) mitochondrial DNA editing, 5) specialized organization of mitochondrial DNA in the kinetoplast, and 6) the sequestration of the glycolytic enzymes in the unique membranous organelle – glycosome [7]. The unique features of *Trypanosoma brucei* render them a popular research object, and their study may help to elucidate fundamental biological phenomena. For this reason, *Trypanosoma brucei* is called the *Escherichia coli* of biochemical parasitology [5]. Several unusual characteristics of *Trypanosomatidae* have already been extensively studied and reviewed in the literature [8], but less attention has been given to membrane energy metabolism in these

organisms. It is only recently, that membrane voltages in *T. brucei* have been investigated in some laboratories [9–14]. The current study describes a method of time-dependent membrane voltage estimation, which is based on the accumulation of TPP^+ by *T. brucei* bloodstream form cells. Changes of TPP^+ concentration in external medium were monitored by selective electrodes elaborated in our laboratory.

MATERIALS AND METHODS

Reagents. Tetraphenylphosphonium chloride was purchased from Aldrich, sodium citrate was from Sigma, D-glucose was from Roth, dithionite was purchased from Riedel-de Haen, all other reagents were of analytical grade.

Trypanosomes. *Trypanosoma brucei brucei* strains WM 14 221 (VSG 221) and WM 17 117 (VSG 117) were 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 [15]. Cells of the long slender form of *T. brucei* were isolated from the blood of Wistar rats three days after infection with $2.5–5 \times 10^7$ cells by intraperitoneal injection. The cells were separated from blood components by centrifugation ($600 \times g$) for 10 min and further purified on a DEAE cellulose column. Washing 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. 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. Cell counts were performed using an SK14 PZO microscope (Poland). Measurements of TPP^+ distribution were performed by selective electrodes as described earlier [16]. The metabolic state of the cells was evaluated by measuring their oxygen consumption with a Clark-type oxygen electrode in a 2 ml chamber of a dual digital oxygen measurement system (Rank Brothers Ltd, England).

RESULTS AND DISCUSSION

The aim of this work was to evaluate and optimize the potentiometric method for estimation of membrane voltages in *Trypanosoma brucei brucei* cell using the lipophilic cation TPP^+ as an indicator of $\Delta\psi$. Recently, membrane voltages in *T. brucei* have been investigated in some laboratories using distribution of radioactive cations or fluorescent probes [9–14]. The first method necessitates separation of the cells from the incubation medium in the withdrawn samples, the tedious procedure that allows to obtain only discrete values of membrane voltages and may distort the results due to the changes in energisation of the cell membranes during centrifugation and/or filtration. The disadvantage of the fluorescent method further extends to the difficulty of obtaining quantitative measurements of $\Delta\psi$ and necessity to calibrate the spectral response of indicator, by imposing K^+ diffusion potentials, for instance [1]. The third method, based on employment of ion-selective electrodes, enables a continuous monitoring of the concentration of $\Delta\psi$ indicator in the medium and the quantitative measurements of $\Delta\psi$ [1, 16]. However, this technique has never been applied to estimate $\Delta\psi$ in trypanosomes so far. Though relatively large amounts of the parasitic cells are required for investigations by this method, they could be easily obtained from the blood of infected rats.

In the following set of experiments the fluxes of TPP^+ across the envelopes of parasite cells were studied at two temperatures (24 °C and 37 °C). In the parallel experiments, metabolic competence of the cells was checked by measuring their respiration rates. The results showed that exposure of the cells to TPP^+ resulted in an intracellular accumulation of the lipophilic cation (Figure, B). It is known that bloodstream form trypanosomes are completely covered by a dense coat of variant surface proteins (VSG) [7]. The VSG dimmers are tightly packed and prevent macromolecules such as complement or immunoglobulin from reaching the membrane. However, our results show that the envelope of *T. brucei* is permeable to TPP^+; thus, there is a sufficient space between the VSG molecules for TPP^+ to reach the plasma membrane. These results are consistent with previous observations of other researchers, who used different radiolabeled lipophilic cations for measurements of $\Delta\psi$ in trypanosomes [10–12, 14, 17, 18]. Equivalent results were obtained using not only *Trypanosoma brucei brucei* strain WM 14 221 (VSG 221), but also with another relative strain, WM 17 117 (VSG 117) (data not shown). Results suggest that trypanosome VSGs neither interfere with the measurements of trypanosome membrane voltages nor affect their magnitudes.

Respiration of the cells was not inhibited by NaN_3, an inhibitor of cytochromoxidase, but was fully blocked by salicylhydroxamic acid (SHAM), as shown in insert of Figure. The results reported here are consistent with the fact that the mitochondrion of long slender bloodstream stage of *T. brucei* lacks cytochromes and classic respiratory chain, but instead contains a SHAM-sensitive plant-like alternative oxidase [19]. This is the unique property of *T. brucei,* and no other cell is known to be solely dependent on an alternative oxidase [19].

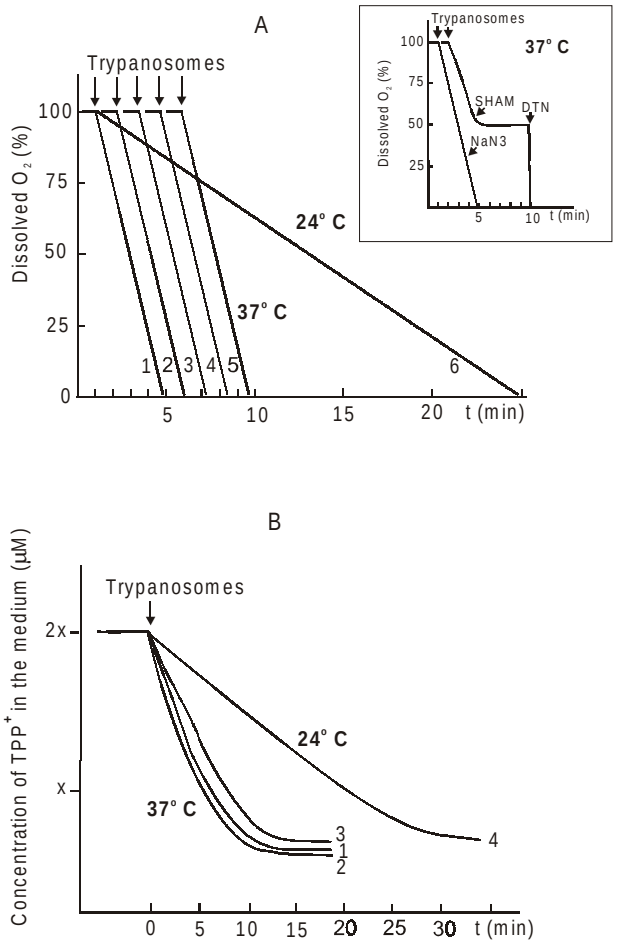

Figure. Effects of incubation temperature and extracellular concentration of TPP^+ on the accumulation of TPP^+ and oxygen consumption by *Trypanosoma brucei brucei* cells. The measurements were performed at 24 °C or 37 °C in trypanosome incubation medium. *Trypanosoma brucei brucei* WM 14 221 (VSG 221) cells were added to the concentration of $2{,}5 \times 10^7$ cells/ml (Panel A) or 2×10^8 cells/ml (Panel B). TPP^+ concentrations were the following: Panel A, curve 2 – 1×10^{-6} M, curve 3 – 2×10^{-6} M, curve 4 – 8×10^{-6} M, curve 5 – 2×10^{-5} M, curves 1 and 6 – without TPP^+; Panel B (for calibration, two additions of x concentration of TPP^+ were made), curve 1 – 2×10^{-6} M, curve 2 – 4×10^{-6} M, curve 3 – 2×10^{-5} M, and curve 4 – $4 \times \times 10^{-6}$ M. Insert: NaN_3 and SHAM were added to the final concentration of 1 mM, a grain of sodium dithionite was added to define the zero level of oxygen in the incubation chamber

Both uptake of TPP^+ and oxygen consumption by *T. brucei* cells were slower at 24 °C than at 37 °C (Figure, A and B). The conclusion can be drawn that at 37 °C *T. brucei* cells are metabolically more active. The steady-state of the distribution of TPP^+ was reached within less than 10 min at 37 °C, but only in about 35 min at 24 °C (Figure, B). These observations are coherent with the fact that a temperature of 37 °C is physiological for the bloodstream form of trypanosomes. Previous studies report that different lengths of incubations (ranging from 10 to 45 min) were required to achieve an equilibrium of the distribution of $\Delta\psi$ indicators. We believe that the reason for this discrepancy was difference in experimental temperatures, which varied from 22 °C to 37 °C [9–12, 14].

Since $\Delta\psi$ indicators are ions themselves, they can disturb the gradients they are used to measure, unless they are used in sufficiently low concentrations to avoid membrane perturbation [1]. Controversial reports exist regarding the action of lipophilic cations, such as TPP^+, on trypanosome viability and membrane voltages. According to Nolan and Voorheis [10], external concentrations of lipophilic cation [^{3}H]MTP^+ higher than 50 nM exert a depolarizing effect on *T. brucei* cells, while Thissen and Wang [14] used a 500 nM final concentration of [^{3}H] TPP^+ without any deleterious effect on the cells. The results of our experiments showed, that at fixed cell concentrations the amounts of TPP^+ in the medium in the range between 2×10^{-6} and 2×10^{-5} M gave a proportional extent of TPP^+ uptake (Figure, B, curves 1, 2 and 3). Microscope examination of trypanosomes showed that the cells were motile and their morphology had not been changed (i.e. possessed identical microscopic appearance with those observed without TPP^+ addition). The respiration rate (Figure, A, curves 2–5) and metabolic activity were also constant throughout incubation for at least half an hour with all concentrations of TPP^+ used. Consequently, at low concentrations employed in this study, TPP^+ neither suppressed the metabolic activity of trypanosomes nor perturbed membrane voltages of the parasitic cell and therefore is valid for potentiometric monitoring of trypanosome $\Delta\psi$. Our further work is intended for estimation of the values of separate membrane voltages in *T. brucei* by an optimized potentiometric method.

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EUKARIOTINIŲ MIKROORGANIZMŲ APVALKALĖLIŲ BARJERINIŲ IR ENERGETINIŲ FUNKCIJŲ ĮVERTINIMAS LIPOFILINIŲ KATIJONŲ PAGALBA. I. *TRYPANOSOMA BRUCEI* MEMBRANŲ ĮTAMPŲ NUSTATYMAS POTENCIOMETRINIU METODU

S a n t r a u k a

Pasiūlytas ir įvertintas potenciometrinis metodas eukariotinių mikroorganizmų *Trypanosoma brucei* membranų įtampoms ($\Delta\psi$) nustatyti $\Delta\psi$ indikatoriais panaudojant lipofilinius katijonus TPP^+. Nustatyta, kad per 30 min. TPP^+ koncentracija iki 2×10^{-5} M nedepoliarizuoja tripanosomų ląstelių ir neslopina jų mitochondrijų kvėpavimo, todėl tinka tripanosomų įtampų potenciometriniams matavimams. Ląstelės kvėpavo ir sugėrė TPP^+ lėčiau esant 24°C negu 37°C. Pastovus TPP^+ pasiskirstymo tarp ląstelės vidaus ir išorės lygis nusistovėdavo per 10 min. esant 37°C ir per 35 min. esant 24°C.