Kinetic study of the plasma-membrane potential in procyclic and bloodstream forms of *Trypanosoma brucei brucei* using the fluorescent probe bisoxonol

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The characteristics of the plasma-membrane potential of procyclic and bloodstream forms of *Trypanosoma brucei brucei* (cultured cells) were investigated using the fluorescent anionic probe bisoxonol. Observation of a stable and representative plasma-membrane potential in the resting state required careful washing, centrifugation and maintenance of the cells at room temperature before measurement. Bloodstream forms were more prone to depolarization during washing at 4 °C than procyclic cells. The higher fluorescence observed in the presence of long slender cells than in the presence of procyclic cells shows that the plasma-membrane potential is more negative in the insect form. Healthy dilute cells can sustain their plasma-membrane potential for hours in the presence of external glucose. The presence of a high K⁺ concentration in the medium did not promote by itself

the depolarization of either type of cell. Study of bisoxonol fluorescence as a function of time allowed us to follow the kinetics of the action of metabolic inhibitors in the presence of various ions. *o*-Vanadate (1 mM) was found to depolarize bloodstream-form cells rapidly but only in a phosphate-free NaCl buffer. Omeprazole and strophanthidin also specifically depolarized bloodstream-form trypanosomes. However, *NN'*-dicyclohexylcarbodi-imide depolarized both types of cell, but more rapidly for bloodstream-form cells. Bloodstream-form trypanosomes appear to use mainly a vanadate-sensitive Na⁺ pump to maintain their Na⁺-diffusion gradient. However, most of the ATPase inhibitors tested had little or no effect on the plasma-membrane potential of procyclics suggesting that this form of trypanosome may rely on several regulation mechanisms.

INTRODUCTION

The African protozoan parasite *Trypanosoma brucei* is the causative agent of sleeping sickness (in humans) and nagana (in domestic animals) and is transmitted by tsetse flies. This parasite has been extensively studied for its variant surface glycoprotein coat and its glucose metabolism. However, it is only recently that several studies have contributed to a better understanding of the bioenergetics of this protozoan. Early studies [1,2] established the existence of a membrane potential. Recently, the transmembrane potentials ($\Delta\Psi$) and the pH gradient (ΔpH), components of the proton electrochemical potential ($\Delta\mu_{\text{H}^+}$), were intensively investigated in various laboratories for the bloodstream form of *T. brucei brucei* using distribution of radioactive cations (Rb⁺, methyltriphenylphosphonium, tetraphenylphosphonium), weak acids and weak bases. Nolan and Voorheis [3–5] established the presence of at least two distinct membrane potentials in the bloodstream form of *T. brucei*: a potential across the plasma membrane ($\Delta\Psi_{\text{p}}$) and a mitochondrial-membrane potential ($\Delta\Psi_{\text{m}}$) which is generated and maintained exclusively by the electrogenic movement of H⁺ across the mitochondrial membrane. The $\Delta\Psi_{\text{p}}$ reported, of the order of –82 mV (inside negative) at 30 °C, seems to be due to the K⁺-diffusion potential. The $\Delta\Psi$ measured by Thissen and Wang [6] is dependent on both the external pH and the external salt conditions. At 22 °C, under ionic conditions similar to those in the host bloodstream, it ranges from –76 to –160 mV over an external pH range of 6.0–8.0 with a value of –155 mV at physiological pH; this $\Delta\Psi$ is the main contributing force to the protonmotive force $\Delta\mu_{\text{H}^+}/F$ (–143 mV under physiological conditions). In a similar study conducted as a function of

temperature, ter Kuile [7] reported that $\Delta\mu_{\text{H}^+}$ and $\Delta\Psi_{\text{p}}$ in the bloodstream form of *T. brucei brucei* vary with the temperature and are significantly lower at 37 °C (–40 mV) than at the subphysiological temperatures of 30 °C [3–5] or 22 °C [6] used in previous studies.

Therefore, even if the existence of a plasma-membrane potential in the bloodstream form of *T. brucei brucei* has been clearly demonstrated, its absolute value has never been established in truly physiological conditions since very different experimental conditions were used by all different workers. Moreover, no information is currently available on $\Delta\Psi_{\text{p}}$, ΔpH and $\Delta\mu_{\text{H}^+}$ in the corresponding procyclic form of the parasite. Furthermore, nothing is known about the regulation systems of these parameters in response to various ionic conditions encountered in the environments characteristic of each host, as well as during their developmental cycle as the trypanosomes have to adapt their metabolism to the passage from one type of host to another.

This report describes the method by which bisoxonol was used to study the membrane potential in bloodstream (*i.e.* long slender) and procyclic forms of *T. brucei brucei* obtained *in vitro*. The aim of this work was to develop, starting from cultured trypanosomes, a protocol allowing the study of the factors governing the plasma-membrane potential using low amounts of *Trypanosoma* cells, under experimental conditions in which it is possible to work with non-toxic probes, as close to physiological conditions as possible and with direct time-dependent monitoring of $\Delta\Psi_{\text{p}}$ changes. The cells were fully motile, respiring and metabolically active throughout our experiments. This fluorescent method using cultured cells also avoids the tedious preparation procedures required to remove blood cells from trypanosomes grown

Abbreviations used: $\Delta\Psi_{\text{p}}$, plasma-membrane potential; ΔpH , transmembrane pH difference; $\Delta\mu_{\text{H}^+}$, electrochemical proton potential; NEM, *N*-ethylmaleimide; DCCD, *NN'*-dicyclohexylcarbodi-imide; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; bisoxonol, bis-(1,3-diethylthiobarbituric acid)trimethine oxonol.

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in rat. Furthermore, all uncertainties and approximations arising from long incubation times, several washes and the determination of intracellular volumes, which are necessary when using radioactive probes, are eliminated.

The only disadvantage of the bisoxonol method is related to the difficulty of obtaining quantitative measurements of transmembrane potentials (in mV). The Na^+/K^+ ionophore gramicidin was used to attempt to obtain a calibration of the electrical potential of cells maintained in a Na^+ -free buffer [8]. Nevertheless, we provide here strong evidence for the usefulness of relative measurements (in arbitrary fluorescence units) to establish which factors are governing the regulation of ionic homeostasis in trypanosomes.

MATERIALS AND METHODS

NaN_3 and KCN were purchased from Merck; *NN'*-dicyclohexyl carbodi-imide (DCCD), *N*-ethylmaleimide (NEM), strophanthidin, sodium *o*-vanadate and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) were from Sigma; ouabain, phloretin, phloridzin, digitonin and gramicidin were obtained from Fluka. Omeprazole was from Research Biochemicals International, and oligomycin from Serva. Bis-(1,3-diethylthiobarbituric acid)trimethine oxonol (bisoxonol) was purchased from Molecular Probes (Eugene, OR, U.S.A.). All other reagents were analytical grade.

Cell cultures

Procyclic forms of *T. brucei brucei* (strain STIB 348 TBABB) were grown as described [9] in SDM-79 medium at 27 °C. Cells were collected after 1 or 2 days of culture, in their growing phase, at a concentration of 6×10^6 to 10^7 cells/ml. Cell density was determined by counting in a Petroff Hausser cell. Bloodstream forms of *T. brucei brucei* (strain STIB 348 TBABB) were grown axenically at 37 °C using MEM 109-4 supplemented with 15 % horse serum in 12- or 24-well cell culture clusters (Costar) [10]. Trypanosomes were collected at 2×10^6 to 3×10^6 cells/ml. All strains were generously provided by Dr. R. Brun (Swiss Tropical Institute, Basel, Switzerland).

Preparation of the cells before measurements

Trypanosomes were washed twice by centrifugation (600 g; 10 min) either in PBS, pH 7.6 (50 mM $\text{Na}_2\text{HPO}_4/3.7$ mM $\text{NaH}_2\text{PO}_4/48$ mM NaCl) supplemented with 56 mM glucose, 5.4 mM KCl and 0.8 mM MgSO_4 (PSGK buffer) or in Hepes SGK, pH 7.5 (40 mM Hepes/41 mM NaCl/56 mM glucose/5.4 mM KCl/0.8 mM MgSO_4). All buffers were sterilized by filtration [cellulose nitrate filters (0.2 μm pore-size; Sartorius) or disposable sterile filters (0.2 μm pores; Schleicher & Schuell)] to remove any contaminants or dust particles. The pellet of cells was then gently resuspended in the washing buffer at a cell density of 5×10^7 to 10×10^7 cells/ml. For the reasons discussed in the Results section, washing and subsequent maintenance of the concentrated cell suspension before measurements were carried out at room temperature.

Fluorescence measurements

Fluorescence was monitored using a Perkin-Elmer LS-5B fluorimeter with excitation and emission slits of 10 nm. Unless otherwise stated, an aliquot of the trypanosome suspension (5×10^6 cells) was diluted in a 1 cm quartz or glass cuvette containing buffer thermostatically controlled at 27 °C for procyclics and 37 °C for the bloodstream forms (final volume 2.5 ml) with magnetic stirring. For most measurements, a Na^+ -rich

buffer was used (Hepes/NaCl: 40 mM Hepes/116 mM NaCl/10 mM glucose/5.4 mM KCl/0.8 mM MgSO_4 , pH 7.5). Hepes/KCl, Hepes/sodium gluconate, Hepes/choline chloride and Hepes/LiCl buffers were occasionally used, where all NaCl was replaced by KCl, sodium gluconate, choline chloride or LiCl respectively. The cuvettes were thoroughly washed using ethanol, conc. HNO_3 and deionized water to eliminate any traces of ionophores.

Changes in plasma-membrane potential were followed using bisoxonol at a final concentration of 100 nM (from a stock solution at 23 μM in 20 % ethanol). Bisoxonol is an anionic fluorescent probe which partitions in the cell membranes and its fluorescence intensity increases as the membrane potential decreases [11,12]. Gramicidin (2.25 μM or less) was used to induce maximal cell depolarization by abolishing the transmembrane gradient of univalent cations. Excitation and emission wavelengths were 535 and 580 nm respectively.

In control experiments, the substances to be tested on the cells were also added to bisoxonol in buffer in order to check that they did not give artefactual fluorescence changes resulting from direct interactions with the dye. The substances were also tested on plasma-membrane vesicles, prepared from trypanosome cells as described elsewhere [13], in the presence of bisoxonol. The concentration of organic solvent in the cuvette was always kept below 1 % to make sure that it did not affect the cells. Occasionally, the metabolic state of the cells was checked by measuring their oxygen consumption using a cell equipped with a Clark-type O_2 electrode.

Calibration of the plasma-membrane potential, $\Delta\Psi_p$, in procyclic cells

Calibration of $\Delta\Psi_p$ was carried out by the procedure recently described for *Leishmania major* promastigotes [8], in Hepes buffer with NaCl replaced by choline chloride. Some 5×10^6 cells were resuspended in 2.5 ml of Hepes/choline chloride buffer, pH 7.5, containing 100 nM bisoxonol, thermostatically controlled at 27 °C. After the addition of the Na^+/K^+ ionophore gramicidin (0.88 μM), increasing concentrations of KCl were added to the medium and the fluorescence increase (ΔF) was recorded. At 27 °C, the transmembrane plasma potential was calculated according to

$$\Delta\Psi_p = -59.4 \log ([\text{K}^+]_{\text{int.}}/[\text{K}^+]_{\text{ext.}})$$

where $[\text{K}^+]_{\text{int.}}$ and $[\text{K}^+]_{\text{ext.}}$ are the internal and external concentrations of K^+ respectively. $[\text{K}^+]_{\text{int.}}$ was taken as 116 mM, the value measured in the bloodstream form [3]. As in [8], it was assumed that $[\text{K}^+]_{\text{int.}}$ remained constant during the brief cell treatment.

RESULTS

Optimization of the method

Preliminary measurements with procyclic-form trypanosomes showed that increasing amounts of cells in the range 10^6 to 10^7 cells/ml in the presence of a constant concentration of bisoxonol (100 nM) gave proportional fluorescence values. At a fixed cell concentration of 5×10^6 cells/ml, the measured fluorescence intensity was also found to be proportional to bisoxonol concentration in the range 50–180 nM (results not shown). All the experiments shown were performed on the same strain (STIB 348 TBABB) for procyclics and bloodstream forms (axenic cultures). Similar results were obtained with the other strains tested (STIB 366 for procyclics, STIB 345 TBABB for bloodstream forms). Moreover, bloodstream forms grown on feeder

Figure 1 Effect of (A) low-temperature treatment on the initial fluorescence of bisoxonol in *T. brucei* bloodstream-form cells and (B) the metabolic inhibitor KCN on the fluorescence of bisoxonol in procyclic cells

(A) For each experiment, 5×10^6 cells were diluted into 2.5 ml of PSGK buffer at 37 °C containing 100 nM bisoxonol; at the time indicated by the arrow, gramicidin (G) (0.9 μ M final concentration) was added to induce maximal depolarization of the cells. (a) Bloodstream-form cells were prepared and kept at room temperature for 1 h; (b) bloodstream-form cells were preincubated for 30 min on ice, then washed and kept at low temperature (0–4 °C) for 20 min; (c) bloodstream-form cells were prepared and kept at low temperature (0–4 °C) for 100 min. (B) Procyclic cells were prepared and kept at room temperature. Some 5×10^6 cells were diluted into 2.5 ml of Hepes/NaCl/5 mM KCl buffer containing 100 nM bisoxonol, at 27 °C. Cells and KCN (4.5×10^{-5} M) were added at the times indicated. a.u., Arbitrary units.

layers of *Microtus montana* embryo fibroblasts as described by Brun et al. [14] showed the same $\Delta\Psi_p$ characteristics as cells grown axenically (results not shown).

The usual experimental conditions were 100 nM bisoxonol and 2×10^6 cells/ml. Since valinomycin interacts directly with bisoxonol in the presence of K^+ [11], gramicidin was used to depolarize the cells completely (Figure 1A). Digitonin (8 μ g/ml for procyclics; 1 μ g/ml for bloodstream form) [7,15,16] and nystatin (procyclics, 12–20 μ g/ml) [4,17], frequently used to permeabilize selectively the plasma membrane of trypanosomes, also induced an increase in fluorescence characterizing depolarization of the cells (results not shown). Bisoxonol, being negatively charged, is known to be excluded from the mitochondrion and to probe selectively the plasma-membrane potential. Indeed, mitochondrial inhibitors such as cyanide (46 μ M; see Figure 1B) [5,15] and oligomycin (2 μ g/ml) [18], which are known to inhibit the respiration in procyclics instantly, had no immediate effect on the bisoxonol fluorescence intensity in procyclics but only a very-long-term metabolic effect. Control experiments in which O_2 consumption of the cells was measured showed that our concentration of KCN instantly inhibited respiration in procyclics (results not shown).

Measurements performed on trypanosomes traditionally washed at 4 °C and subsequently kept on ice showed a marked initial increase in fluorescence intensity on addition of the cells followed by a decrease characterizing a partial repolarization of the cells (Figure 1A, traces b and c). However, cells washed and kept at room temperature showed a steady and reproducible fluorescence intensity from the beginning of the measurement (Figure 1A, trace a). Bloodstream-form cells were found to be

Figure 2 Calibration of the plasma-membrane potential ($\Delta\Psi_p$) in the procyclic form of *T. brucei brucei*

The inset shows the variation in fluorescence of 100 nM bisoxonol (excitation, 535 nm; emission, 580 nm) in Hepes/choline chloride buffer at 27 °C on addition of 5×10^6 cells, followed by 0.88 μ M gramicidin (G) and then addition of increasing concentrations of KCl (2.5, 5, 7.5, 12.4, 22.3, 32.1, 51.4, 70.5, 89.2, 116.8, 125.8 mM, final concentration). The main graph depicts the relationship between bisoxonol fluorescence change (ΔF) and the calculated K^+ equilibrium potential, $\Delta\Psi_p$, in mV (for details, see the Materials and methods section and ref. [14]). The graph is representative of seven different experiments. a.u., Arbitrary units.

more sensitive to low temperatures than procyclics and their depolarization on ice was often irreversible (Figure 1A, trace c). Consequently, in order to maintain the transmembrane potential in trypanosomes, the cells were always washed and kept at room temperature in a sterile glucose-rich buffer until use. Bloodstream forms could be maintained at room temperature for up to 1.5 h and procyclics for about 3 h; cells remained fully motile throughout our experiments.

Calibration of the plasma-membrane potential ($\Delta\Psi_p$) in procyclics

The calibration of $\Delta\Psi_p$ in mV in procyclic cells is shown in Figure 2. The measurements were based on the establishment of known ionic diffusion potentials of K^+ in a Na^+ -free buffer in the presence of gramicidin. In the choline chloride buffer used, the resting potential estimated from the calibration curve was -121 ± 2 mV in the presence of gramicidin and, by extrapolation, -152 ± 5 mV before the addition of the ionophore. However, these calibration experiments showed that the cells repolarize at higher K^+ concentrations. The absence of complete depolarization of the cells by K^+ made it impossible to obtain a calibration curve that was precise enough in the low electrical potential (i.e. high fluorescence) range. Consequently, in the present study, the fluorescence scale (in arbitrary units) was preferred to a $\Delta\Psi_p$ scale in mV.

Resting membrane potential in both forms of *T. brucei*

The steady state of fluorescence intensity of bisoxonol on addition of the same number of cells was significantly higher for bloodstream forms than for procyclics. The difference cannot be explained by a direct effect of temperature on the probe (measurements performed at 27 °C for procyclics, and 37 °C for bloodstream forms) since the fluorescence intensity is known to decrease when the temperature is increased. Moreover, such a difference was maintained if both types of cell were studied at the same temperature. Consequently, the higher fluorescence intensity observed in the presence of bloodstream cells corresponds to a lower membrane potential. However, attempts at calibration

Figure 3 Effect of 1 mM sodium *o*-vanadate on the fluorescence of bisoxonol in *T. brucei* cells

Cells (bloodstream form a–d, procyclic form, e) were maintained in different types of iso-osmotic buffer. Bisoxonol (100 nM) was preincubated in buffer at 37 °C (a–d) or 27 °C (e) before addition of trypanosomes (first arrow, final concentration 2×10^6 cells/ml) and *o*-vanadate (second arrow). Bloodstream-form cells were resuspended in Hepes buffer containing NaCl (a), LiCl (b), KCl (c) and sodium gluconate (d). For trace (e), procyclic cells were resuspended in Hepes/NaCl buffer. Traces are representative of at least two different experiments. Other experimental conditions are described in the Materials and methods section. a.u., Arbitrary units.

Figure 4 Effect of NEM and omeprazole on the fluorescence of bisoxonol (580 nm) in procyclic- and bloodstream-form *T. brucei* cells

Bisoxonol (100 nM) was preincubated in Hepes/NaCl buffer at 27 °C for procyclic cells (traces a and c) and at 37 °C for bloodstream cells (traces b and d). NEM was added at a final concentration of 1 mM to procyclics (a) and 0.5 mM to bloodstream forms (b). Omeprazole (Omepr.) was added at a final concentration of 0.4 mM to procyclics and 0.2 mM to bloodstream-form cells (d). Traces are representative of at least two different experiments. Other experimental conditions are described in the Materials and methods section. a.u., Arbitrary units.

performed on bloodstream-form cells at 37 °C did not allow the determination of $\Delta\Psi_p$ in this form of trypanosome. Bloodstream-form cells appeared to be completely depolarized by 0.88 μ M gramicidin in KCl-free Hepes/choline chloride buffer. Using only 0.2 μ M gramicidin, it was possible to obtain a stepwise depolarization of the cells with KCl but it induced a rapid marked repolarization of the cells, even at low K^+ concentrations (results not shown).

Both types of cell, procyclic and bloodstream, gave very steady

Table 1 Effect of ATPase inhibitors on the $\Delta\Psi_p$ of procyclic- and bloodstream-form *T. brucei* cells

+ +, Rapid depolarization; +, slow depolarization; —, no effect on $\Delta\Psi_p$.

Inhibitor	ATPase type	Concentration	Bloodstream	Procyclic
<i>o</i> -Vanadate	P-type	1 mM	+ + (in NaCl)	—
Omeprazole	H ⁺ , K ⁺	200 μ M	+ +	—
Ouabain	Na ⁺ , K ⁺	0.5 mM	—	—
Strophanthidin	P-type	0.4 mM	+	—
DCCD	H ⁺	20 μ M	+	+

fluorescence traces over long periods of time in Hepes/NaCl buffer (Figure 3, traces a and e). However, bloodstream cells that had undergone osmotic swelling or kept without glucose were highly depolarized and did not recover their normal potential on addition of energy sources. Similarly, bloodstream cells with an abnormally high fluorescence at the beginning of an experiment, indicating partial depolarization, continued to depolarize spontaneously in the cuvette (results not shown).

These control experiments showed that bisoxonol is a very sensitive probe for specifically monitoring the plasma-membrane potential of both forms of trypanosomes. Moreover, it was found that cells prepared and kept at room temperature were able to maintain a steady potential over long periods of time. Subsequently, these optimized conditions were used to investigate the variations in $\Delta\Psi_p$ in both forms of *T. brucei* in the presence of various inhibitors.

Effect of different buffers, vanadate and NEM

Figure 3 (traces a–d) shows the bisoxonol fluorescence intensity in the presence of bloodstream-form cells in different types of buffer, followed by the effect of 1 mM sodium *o*-vanadate, an inhibitor of P-type ATPases. In Hepes/NaCl buffer, the potential of the cells was very steady but addition of *o*-vanadate resulted in rapid depolarization of the cells (Figure 3, trace a). Vanadate had no effect if Hepes/NaCl buffer was replaced by a phosphate/NaCl buffer (results not shown). When NaCl was replaced by KCl, LiCl or sodium gluconate in phosphate-free buffers, the bloodstream-form cells tended to depolarize very slowly, especially in LiCl, and subsequent addition of vanadate increased slightly the rate of cells depolarization. These results suggest competition of vanadate with phosphate at the level of a P-type ATPase and indicate that this inhibition requires the presence of both Na^+ and Cl^- ions in the external buffer to result in depolarization of the cells. The gradual exchange of Na^+ for K^+ in the external buffer (Cl^- concentration being kept constant) modified both the kinetics and the extent of depolarization induced by vanadate. A marked decrease in the inhibitor's effect was observed for Na^+ concentrations lower than 50 mM (results not shown). In similar experiments, the potential of procyclics remained very steady in Hepes buffer containing either NaCl, KCl, LiCl or sodium gluconate, and subsequent addition of 1 mM vanadate had no effect in any of these buffers (see Figure 3, trace e for Hepes/NaCl). Thus, in conditions where the regulation of the plasma-membrane potential of bloodstream-form cells was sensitive to vanadate, procyclic cells were not at all affected. These experiments also showed that, at resting potential,

Figure 5 Effect of ATPase inhibitors on the fluorescence of bisoxonol in procyclic- and bloodstream-form *T. brucei* cells

Bisoxonol (100 nM) was preincubated in Hepes/NaCl buffer at 27 °C for procyclic cells (traces a, c and e) and at 37 °C for bloodstream-form cells (traces b, d and f). Ouabain (a, b) and strophantidin (Stroph.) (c, d) were added to obtain final concentrations of 0.5 mM and 0.4 mM respectively. DCCD was added at a final concentration of 40 μ M to procyclic (e) and 20 μ M to bloodstream-form cells (f). Traces are representative of at least two different experiments. Other experimental conditions are described in the Materials and methods section. a.u., Arbitrary units.

Figure 6 Effect of glucose-transport inhibitors (phloretin and phloridzin) on the fluorescence of bisoxonol in procyclic and bloodstream *T. brucei* cells

Bisoxonol (100 nM) was preincubated in Hepes/NaCl buffer at 27 °C for procyclic cells (traces a and c) and at 37 °C for bloodstream-form cells (traces b and d). Phloretin was added at a final concentration of 130 μ M to procyclics (trace a) and 20 μ M to bloodstream forms (trace b). Phloridzin was added at a final concentration of 2.5 mM to both types of cell (traces c and d). Traces are representative of at least two different experiments. Other experimental conditions are described in the Materials and methods section. a.u., Arbitrary units.

a K^+ -rich buffer does not affect the potential of trypanosomes (Figure 3, trace c for bloodstream forms).

Addition of 1 mM NEM induced a rapid increase in bisoxonol fluorescence in bloodstream forms either in NaCl (Figure 4, trace b) or in KCl (results not shown) buffers. Such a strong effect of NEM on bloodstream forms was also reported by Thissen and Wang [6]. However, procyclics were only very slowly depolarized by NEM (Figure 4, trace a).

Other carrier inhibitors

Figure 4 (traces c and d) shows that addition of the H^+/K^+ -ATPase inhibitor omeprazole (200 μ M) to bloodstream-form cells induced a marked depolarization whereas procyclics were not affected at all, even with a higher concentration of the inhibitor (400 μ M).

The Na^+/K^+ -ATPase inhibitor ouabain (0.5 mM) did not

modify the resting potential of procyclics or bloodstream-form trypanosomes (Figure 5, traces a and b) even in KCl-free Hepes/NaCl buffer (results summarized in Table 1).

Strophanthidin (400 μ M), which is also a digitalis-derived inhibitor of P-type ATPases but less specific than ouabain [19], induced slow depolarization in bloodstream-form trypanosomes (Figure 5, trace d). The effect of strophanthidin was slightly faster in NaCl than in KCl buffer (results not shown). However, strophanthidin did not affect $\Delta\Psi_p$ in procyclics (Figure 5, trace c).

Bloodstream-form cells were also found to be depolarized by DCCD (40 μ M), an H^+ -ATPase inhibitor (Figure 5, trace f). The effect of DCCD on procyclics was different, inducing mainly a rapid but only partial depolarization followed by a very slow increase in fluorescence intensity (Figure 5, trace e). Addition of oligomycin (1 μ g/ml) or FCCP (0.12 μ M) did not interfere with the inhibition by DCCD suggesting that DCCD mainly inhibits a non-mitochondrial ATPase.

Two inhibitors of glucose transport were tested on bloodstream and procyclic trypanosomes (Figure 6). Phloretin (0.14 mM), an inhibitor of facilitated-diffusion glucose transporters [20], induced a clear depolarization of bloodstream-form cells; no immediate effect could be observed on procyclics. An inhibitor of Na^+ -dependent glucose transport, phlorizin (2.5 mM), had no inhibitory effect on either type of cell.

DISCUSSION

Trypanosoma cycles between an insect vector (procyclic form) and a mammalian host (bloodstream form). The way in which the parasite regulates its ion-transport mechanisms and adapts to its different hosts is still poorly understood. This is due to the lack of studies on the procyclic form and also to the fact that most studies have been performed at non-physiological temperatures. This study contributes to a better understanding of the factors governing the plasma-membrane potential; it exploits the sensitivity of fluorescence spectroscopy to monitor quick variations in $\Delta\Psi_p$ using low concentrations of fully motile cells.

Bisoxonol, a voltage-sensitive dye which is non-toxic and excluded from mitochondria [12,21], has been successfully used to study the plasma-membrane potential in various types of cells including lymphocytes [22], mast cells [12] and, more recently, *T. brucei brucei* [23] and *L. major* [8]. The rapid equilibration of bisoxonol with trypanosomes (less than 30 s) allowed us to demonstrate that handling of the cells at room temperature prevented disturbance of their resting potential. Indeed, low-temperature treatments induced depolarization of trypanosomes, bloodstream forms being more sensitive than procyclics. Lonsdale-Eccles and Grab [24] suggested that placing bloodstream-form trypanosomes on ice affects the morphology and biochemistry of the parasite but very few authors maintain their trypanosomes at room temperature before measurements [23]. Maintenance of the cells on ice could explain the apparent contradiction between our results and the observation by ter Kuile et al. [7] that $\Delta\Psi$ in bloodstream forms of *T. brucei brucei* falls dramatically when the temperature increases. Obviously, further work is necessary to understand more clearly the influence of temperature on $\Delta\Psi_p$ and $\Delta\Psi_m$.

Very few calibration methods have been described for correlating bisoxonol fluorescence intensity and potential in mV [8,22]. Under the experimental conditions successfully used for *L. major* promastigotes [8], bloodstream forms and, to a lesser extent, procyclics prevented accurate calibration by activating ionic regulation systems at high K^+ concentrations. Therefore

the $\Delta\Psi_p$ of -152 mV calculated in procyclics is only an estimate, but it is close to the values measured in bloodstream forms at 20 °C by other workers [6,7].

Studied at their respective physiological temperatures, the $\Delta\Psi_p$ in the procyclic form, which has never been investigated before, was found to be more negative than in the bloodstream form. This is consistent with the observations made by Divo et al. [18] as interpreted by ter Kuile [25].

Procyclic cells were found to maintain their resting potential independently of the univalent cations used in the buffer (Na^+ , K^+ , Li^+). Bloodstream forms depolarized but at an extremely slow rate in $LiCl$ - and KCl -rich buffers. This last observation shows that there is no passive electrogenic K^+ channels active at the resting potential and does not explain the accumulation of Rb^+ observed in a Na^+ buffer in the absence of valinomycin [3,5], nor the less negative potential measured in KCl buffer [6]: -90 mV instead of -150 mV in $NaCl$ buffer. Again, comparisons are made difficult by the different times and temperatures of incubation chosen by these authors (22 and 30 °C; 10 and 45 min).

For the first time, bloodstream-form cells have been shown to be quickly depolarized by vanadate in $NaCl$ buffer, which suggests the presence of an ATP-dependent pump maintaining the Na^+ -diffusion potential. Surprisingly, inhibition requires the presence of both Na^+ and Cl^- . As vanadate cannot permeate the membrane, it must somehow enter the cell to be active on the cytoplasmic side [26], for instance via an anionic channel or carrier which may explain the Cl^- -dependence of this process. Trypanosomes were found to be resistant to high concentrations of the Na^+ , K^+ -ATPase inhibitor ouabain. Nolan and Voorheis [3] measured a very small inhibition effect of ouabain on the distribution of $^{86}Rb^+$ in bloodstream-form *T. brucei* cells. These results suggest that the vanadate-sensitive Na^+ pump is ouabain-insensitive in this parasite. Ouabain-resistance of Na^+ pumps has been described previously [27,28] and can be confirmed by single mutations of the Na^+ , K^+ -ATPase [29].

The $\Delta\Psi_p$ of bloodstream-form cells was also reduced by strophanthidin, an aglycone equivalent of ouabain which has been shown to inhibit H^+ -, Ca^{2+} - and H^+ , K^+ -ATPases [19], and also by omeprazole, an inhibitor of gastric H^+ , K^+ -ATPases, which are both membrane permeant. Since omeprazole is only activated at low pH [30], it may, as a weak base, accumulate in acidocalcisomes, the acidic compartments found in trypanosomes [16] and, after activation, react rapidly with the thiol group of enzymes, possibly a Ca^{2+} , H^+ -ATPase system, inducing rapid depolarization of the bloodstream-form trypanosomes. DCCD seemed to inhibit a plasma-membrane H^+ -ATPase present in both forms. A DCCD-sensitive H^+ -pump was also shown in *L. major* promastigotes [8]. Further work is in progress to gain a better understanding of the intracellular pH regulation systems in *T. brucei brucei*.

The bloodstream forms of *T. brucei brucei* are totally dependent on D-glucose for their energy supply [31]. Indeed, cells that had exhausted the glucose present in the medium (after 2–3 h under our experimental conditions) were depolarized (results not shown). Two inhibitors of glucose transport were tested and only phloretin depolarized bloodstream cells. This observation agrees with the earlier observation [32] of glucose transport by facilitated diffusion in the bloodstream form, which is now generally accepted [25]. The mechanism of glucose transport in procyclics is still under debate: active transporters and facilitated diffusion carriers have been reported [25]. The absence of any obvious effect of these inhibitors on the procyclics may also be due to energy reserves allowing these cells to maintain their electrical potential.

In conclusion, the present study has shown that the blood-stream form of trypanosomes is readily depolarized by several ATPase inhibitors, and also by factors such as temperature changes and lack of glucose. These observations are in good agreement with cells living in the bloodstream of mammals, a very steady biological environment. However, procyclic cells, which normally live in the midgut of tsetse flies, seem to be very resistant to ionic and temperature changes. Most of the ATPase inhibitors tested had no effect on the $\Delta\Psi_p$ of intact procyclic cells, and a complementary study, in progress, indicates that these inhibitors do not affect the ATPase activity in plasma-membrane microsomes extracted from procyclics. Further work is necessary to characterize more fully the pumps involved in the regulation of ionic homeostasis in *T. brucei*, but our results indicate that some of these ionic pumps would be appropriate targets for trypanocidal drugs.

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