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Effects of high external calcium concentrations on etiolated *Euglena gracilis* Z cells and evidence of an internal membrane potential

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K^+ and Cl^- internal concentrations and the total internal Ca^{2+} pool have been determined in *Euglena gracilis* cells. Membrane potentials and resistances were determined using microelectrodes that were inserted into individual *Euglena* cells held at the tip of a holding micropipette by means of suction. A mean plasma membrane-potential difference of −58 mV was observed in accordance with previous results on other microorganisms, a finding which, together with the calculated equilibrium potential of Ca^{2+}, suggests that this organism extruded Ca^{2+} against the thermodynamic gradient. An internal compartment membrane-potential difference of +38 mV, positive inside, was measured. Variation of the external calcium concentration resulted in changes of these membrane potentials. Increasing the external calcium concentration depolarised (made less negative) the plasma membrane potential but polarised (made more positive) the internal compartment membrane potential. The nature of this internal compartment is discussed.

Introduction

The unicellular alga *Euglena gracilis* is a non-vacuolated microorganism which has been studied intensively from the biochemical and physiological point of view [1–3]. Membrane and ion transport studies are rather less common on this organism because they require the measurement of the electrical potential difference across the cell surface. Obtaining such a parameter in this organism presents serious difficulty because of cell dimension and motility. Nevertheless, it has been possible to devise a method of inserting microelectrodes into single *Euglena gracilis* cells according to methods already applied on *Chlorella* [4], *Paramecium* [5], plant cells, protoplasts [6–9] or tonoplast vesicles [10]. The experiments were conducted under fixed external conditions such that the measured potential difference could be correlated with the distribution of ions.

Previous results assigned a stabilizing role to a high external calcium concentration (100 mM) on calcium alginate-immobilized [11] or free *Euglena* cells [12]. On the hypothesis that this stabilization may be due to an internal redistribution of ions and an internal accumulation of calcium, we studied the possible correlation between various external calcium concentrations and the measured potential differences.

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Materials and Methods

Organism and culture conditions. Etiolated *Euglena gracilis* cells (strain Z (25) Pringsheim) were grown organotrophically at 25°C in a defined synthetic medium (L medium) containing 33 mM D,L-lactate as the sole usable carbon source at pH 3.5 [13].

Storage procedure of cells. Cells were stocked in darkness at 4°C in different storage media. Each storage medium (SM(i)) consists of $CaCl_2$ at the final concentration of i M in distilled water at the final pH of 5.5. For instance, SM(0.01) medium is a 0.01 M $CaCl_2$ solution.

Cell number. Cells were fixed with 10% (w/v) KI and counted on a Malassez counter chamber. At least three counts have been averaged to determine each cell number.

Measurements of ionic concentrations

Ion extraction. Cells growing in culture medium or stored in storage media were sampled and centrifuged at 1500 × g for 10 min. Pellets were washed three times with twice-distilled water and resuspended in a 0.1 M HNO_3 extraction medium. After a 48–72 h storage, this extraction medium was centrifuged and ions were measured in the supernatant.

Measurement of calcium ion. Calcium concentrations were measured by an atomic absorption spectrophotometric method with a IL 353 Photometer. Samples were diluted in a 0.5% lanthane + 5% HNO_3 solution in order to offset interference by other ions.

Measurement of chloride ion. Chloride concentrations were measured by a colorimetric method using mercuric thiocyanate iron and ammonium alum. Light absorption at 505 nm by iron thiocyanate was measured.

Measurement of potassium ion. Potassium concentrations were measured by an atomic emission photometric method using a Technicon autoanalyser. Samples were diluted in a $LiNO_3$ solution.

Measurement of cellular potential difference and resistance

Preparation of the holding micropipette. Because of its small size and mobility and the elasticity of its external pellicle, *Euglena* cells were drawn onto the tip of a glass holding micropipette by means of suction from a syringe. The holding micropipette was drawn from a glass capillary tube by an electrode puller (Narishige). Using a microforge (De Fonbrune), the tip of the capillary tube was heated to form a very small ball (approx. 10 to 20 μm outer diameter) which, after being cooled, was then carefully cut by a heat shock into a hemispherical cup.

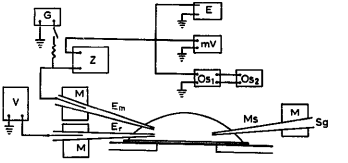

Fig. 1. Laboratory-made wiring diagram for measuring cellular potentials and resistances. E, recorder; Em, glass microelectrode; Er, reference microelectrode; G, frequency generator; M, micromanipulators; Ms, holding micropipette; mV, electrometer; Os_1 and Os_2, oscilloscopes, for potentials and resistances, respectively; Sg, pressure syringe for holding the cell; V, calibration box; Z, impedance adaptator.

Preparation of the glass microelectrodes. The glass microelectrodes were drawn from glass capillary tubes with a glass fiber inside (Clarck, GC 200 F) by an electrode puller (Narishige) and filled with 3 M KCl.

Measurement of electrical membrane-potential difference (interior related to medium). High-impedance glass microelectrodes (30 to 50 MΩ) with low tip potential (less than 5 mV in distilled water), connected via Ag/AgCl contacts to an electrometer (input impedance = 5000 MΩ) and recording devices (oscilloscope, line recorder) were applied in the usual way (see the wire-diagram of Fig. 1). All the recordings were determined at room temperature of about 20°C. The horizontally oriented microelectrode was gently and slowly pushed into the cell to obtain a good puncture.

Measurement of electrical membrane resistance. The 'one-electrode' technique was used according to Ref. 14 and 15 because it was extremely difficult to insert more than one electrode (tip 0.5 μm) into *Euglena* cells (size 15 × 50 μm). The

difficulties of the method have been discussed by Etherton et al. [15]. The membranes and the glass microelectrode can be analogous to a RC circuit. A Tektronic PG 505 pulse generator injected a constant current and a square signal (pulse height of 4 nA with a form factor of 50%) of variable frequency (1 to 10^6 Hz) through the recording microelectrode. A frequency of 10 kHz was choosen so that the relation $T_m > 10^4\ s > T_e$ (T_m = membrane time constant and T_e = microelectrode time constant) was satisfied. This technique allowed us to follow the penetration of the microelectrode through the different membranes, using the Ohm's law of resistances in series. So, with some precautions, the measurement of the resistance estimated the degree of cellular compartmentation.

Results

Intracellular concentrations

Cell volume was measured with a haemocytometer and its mean value ± S.D. (n = number of experiment), $v_i = 1.1 \pm 0.2$ (5) pl per cell, was used to determine the internal concentration of K^+ and Cl^- assuming that these ions were in free solution and not cytoplasmically bound. Considering that Ca^{2+} is highly bound to membranes and that its free cytoplasmic concentration is highly regulated [16–18], this generally accepted value is around 10^{-7} M [18–20]. Table I presents the internal concentrations of K^+, Cl^- and Ca^{2+} of cells growing on L medium.

When cells were transferred and adapted to the storage medium SM(0.1), changes in internal ionic composition occurred and are given in Table II. Ca^{2+} and Cl^- content increased, whereas the K^+ pool decreased.

Electrical measurements

External membrane-potential difference and resistance under fixed external conditions

The mean potential difference and resistance obtained from impaling *Euglena* cells in L medium are given by the equations:

$$E_m = -58 \pm 5\ (47)\ \text{mV}, \quad R_m = 48 \pm 8\ (22)\ \text{M}\Omega$$

TABLE I

THE DISTRIBUTION OF IONS UNDER FIXED CONDITIONS

The value of the internal levels of K^+ and Cl^- are as determined for cells cultured in L medium at 25°C although Ca^{2+} level corresponds to generally accepted value from references (Campbell, 1983). The values are quoted as mean ± S.D., are expressed as mmol·l^{-1}, and the figures given in parentheses represent the number of experiments. The external concentrations of the three ions are given and also expressed as mmol·l^{-1}.

Ion	External concentration (C_o)	Internal concentration (C_i)
K^+	4.4	98.6 ± 4.9(5)
Cl^-	3.65	6.1 ± 0.5(5)
Ca^{+2}	2.0	0.0001

These values represent results recorded from successful puncture that gave sharp jumps to steady potential, usually for 10–60 s, followed by the removal of the microelectrode and an immediate decrease to the original tip potential.

Evidence of intracellular potential difference and resistance under fixed external conditions.

For the recording of transcellular electrical profile, the microelectrode was gently and slowly pushed through the cell until it came to the site opposite the entry of the microelectrode [8]. A profile was considered to be successful if the original tip potential was obtained at the end of the

TABLE II

CHANGES IN INTERNAL IONIC COMPOSITION DURING ADAPTATION OF *EUGLENA* CELLS FROM CULTURE MEDIUM TO THE 100 mM Ca^{2+} STORAGE MEDIUM

The values are quoted as mean ± S.D., are expressed as fmol per cell and correspond to the mean value of five experiments. The charge transfer indicates for each ion the quantity of electrical charge transferred into the cell and is expressed as fmol equivalent electron per cell.

Ion	Internal quantity (fmol·cell^{-1})		Charge transfer $Z(C_{i_2} - C_{i_1})$
	L medium (C_{i_1})	SM (0.1) medium (C_{i_2})	
K^+	98.6 ± 4.9	85.7 ± 4.7	−12.9
Cl^-	6.1 ± 0.5	19.2 ± 2.1	−13.1
Ca^{2+}	1.6 ± 0.2	15.1 ± 1.3	+27

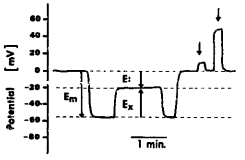

Fig. 2. Example of a transcellular electrical profile. This profile was obtained by the electrode penetrating a *Euglena gracilis* cell. The first values of electrical potential E_m (−58 mV) corresponds to the plasma membrane while the second value E_i (−20 mV) corresponds to the penetration of the microelectrode into an internal compartment, positive inside compared to the cytoplasm ($E_x = E_i - E_m = +38$ mV). Note that the two arrows indicate, respectively, a 10 mV and a 50 mV signal for calibration.

experiment, i.e., when the microelectrode either had crossed the cell or was removed from the cell. Surprisingly, a positive inside (compared to the cytoplasm) intracellular potential difference was sometimes obtained (Fig. 2). The mean values of this potential difference and resistance, if compared to the L medium, are given by:

$E_i = -20 \pm 4$ (13) mV and $R_i = 130 \pm 14$ (9) MΩ

or, if compared to the cytoplasm:

$E_x = +38 \pm 7$ mV

and $R_x = 72 \pm 18$ MΩ ($E_x = E_i - E_m$, $R_x = R_i - R_m$)

Potential differences under different external calcium concentrations

The same type of experiment was then applied to *Euglena* cells adapted to different storage media, i.e. to different external calcium concentrations. The results, summarized in the Fig. 3, exhibit a direct correlation not only between the external membrane potential difference and the external calcium concentration (Fig. 3a) but also between the intracellular potential difference and the external calcium concentration (Fig. 3b). We have to notice that in some cases the $CaCl_2$ medium was replaced by a $CaCO_3$ medium without changing the value of the membrane potential obtained. This indicates that Ca^{2+} and not Cl^- is involved in these experiments.

Discussion

These results are the first reported measurements of the membrane potentials of *Euglena gracilis* Z cells. When one deals with cells of small dimension, the effect of salt leakage from the microelectrode is an obvious factor. Indeed, KCl leakage would induce a hyperpolarization of the measured potential followed by a final depolarization, and observation which would indicate cellular death due to the deleterious effect of high concentration of K^+. This event was not observed except in a few experiments when the tip of the microelectrode was disturbed. The mean value of −58 mV is quite acceptable in comparison to the values of membrane potential in other nonvacuolated microorganisms.

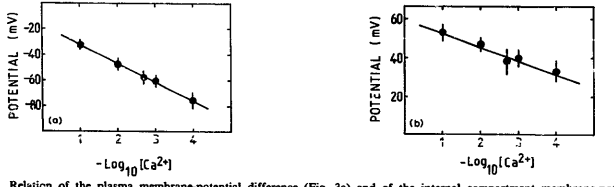

Fig. 3. Relation of the plasma membrane-potential difference (Fig. 3a) and of the internal compartment membrane-potential difference (Fig. 3b) to the extracellular Ca^{2+} concentration. The potential differences are expressed as mV and Ca^{2+} concentrations as mol·l^{-1}. The bars indicate ±S.D.

Adaptation of *Euglena* cells to various external calcium concentrations (from a 2 mM to a 100 mM medium) induced an increase of up to 10-fold of the total intracellular calcium pool. How does calcium enter the cells? What happens to the entered calcium? Is it all or partly sequestered? Where is it sequestered?

Some speculation about the occurrence and direction of transport processes in *Euglena* can be drawn from the knowledge of the plasma membrane potential together with values of ionic concentrations. The equilibrium potential for K^+, Cl^-, and Ca^{2+} have been calculated using the Nernst equation and are given in Table III. All the three values do not agree with the measured potential. Since we do not know whether *Euglena* cells are in flux equilibrium for each of these ions, we cannot ascertain if there are metabolic pumps maintaining the constant internal ionic composition. The calculated electrochemical force of calcium that is directed from the bathing medium to the cytoplasm is so enormous ($\Delta E = -154$ mV) that it could be maintained only if there were a calcium pump, or if this huge calcium gradient were directly coupled to another ion gradient, which itself

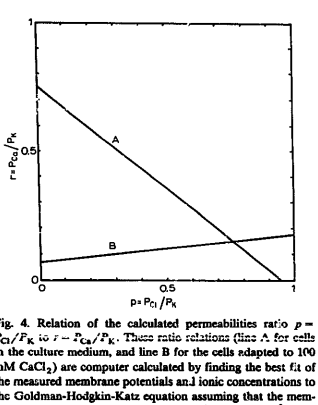

Fig. 4. Relation of the calculated permeabilities ratio $p = P_{Cl}/P_K$ to $r = P_{Ca}/P_K$. These ratio relations (line A for cells in the culture medium, and line B for the cells adapted to 100 mM $CaCl_2$) are computer calculated by finding the best fit of the measured membrane potentials and ionic concentrations to the Goldman-Hodgkin-Katz equation assuming that the membrane current is mainly carried by K^+, Cl^-, and Ca^{2+}.

TABLE III

THE EQUILIBRIUM POTENTIALS ARE CALCULATED FROM THE NERNST EQUATION USING THE INTERNAL CONCENTRATIONS GIVEN IN TABLE I AND ARE EXPRESSED AS mV

Ion	$\frac{C_o}{C_i}$	$E = \frac{58}{Z} \log \frac{C_o}{C_i}$
K^+	0.045	−78
Cl^-	0.579	+13
Ca^{2+}	20000	+125

were supplied with the energy necessary to maintain it far from the calcium equilibrium potential. Because K^+ or Cl^- gradient cannot play this role, the existence of a passive entry of calcium and of a calcium pump on the plasma membrane of *Euglena* cells is the most probable event.

If the Goldman-Hodgkin-Katz equation [21,22] is applied to the membrane potential of etiolated *Euglena* cells, assuming that the membrane current is mainly carried by K^+, Cl^- and Ca^{2+}, then

$$E_m = RT/F \ln[A + (A^2 + 4BC)^{1/2}/2C]$$

with $A = [K^+]_o - [K^+]_i + p([Cl^-]_i - [Cl^-]_o)$, $B = [K^+]_o + p[Cl^-]_i + 4r[Ca^{2+}]_o$ and $C = [K^+]_i + p[Cl^-]_o + 4r[Ca^{2+}]_i$, where $p = P_{Cl}/P_K$ and $r = P_{Ca}/P_K$, respectively, are the ratios of the ion permeabilities of the membrane for Cl^- and K^+ and for Ca^{2+} and K^+, $[K^+]_o$ and $[K^+]_i$, $[Cl^-]_o$ and $[Cl^-]_i$, $[Ca^{2+}]_o$ and $[Ca^{2+}]_i$, respectively, are the concentrations of K^+, Cl^- and Ca^{2+} outside and inside the cell. $[Ca^{2+}]_i$ was in the accepted range 10^{-7}–10^{-5} mol·l^{-1}, $[K^+]_i$ and $[Cl^-]_i$ were measured, $[K^+]_o$, $[Cl^-]_o$ and $[Ca^{2+}]_o$ were known. The best fit of the calculated value of E_m to the experimental data was found by adjusting the value of the two ratios. This ratio relation ($r = f(p)$) is described as a line in Fig. 4 for cells in the usual 2 mM Ca^{2+} culture medium (A) and for cells adapted to 100 mM Ca^{2+} medium (B). The fact that the usual values previously reported for p are in the range 0.02–0.1 means that r is relatively high in the culture medium (from 0.67 to 0.75). This ratio is decreased after adaptation to 100 mM Ca^{2+} storage medium, down to the range 0.07–0.08 if we consider p to be unaffected. If

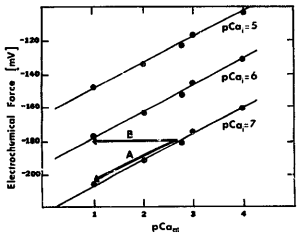

Fig. 5. Relation of the calculated electrochemical force of Ca^{2+} across the plasma membrane ($\Delta E = E_{Ca} - E_m$) to the extracellular Ca^{2+} concentration. The value of the cytoplasmic free calcium concentration is indicated for each curve. The adaptation of *Euglena* from a 2 mM to a 100 mM Ca^{2+} medium induces either an increase in the electrochemical force (see arrow A) or a change in the cytoplasmic free calcium concentration (see arrow B).

not, we have to assume that p is increased to the range value 7–8, a highly improbable event. Such a high calcium permeability (compared to K^+ permeability) in the 2 mM medium may explain why calcium ions entered the cell when the external calcium concentration suddenly increased 50-fold. The cells are adapting to such a high calcium medium by decreasing the calcium permeability (compared to the K^+ permeability) of their plasma membrane.

The role of choride ions is not negligible since variations in cellular chloride content occurred. Nevertheless, they are not so much important since we showed that $CaCO_3$ in the external medium nearly induced the same effects on external membrane potential than $CaCl_2$.

The calculated electrochemical force on calcium across the plasma membrane ($\Delta E = E_{Ca} - E_m$) was studied as a function of the external calcium concentration. The results for $[Ca^{2+}]_i$ from 10^{-7} to 10^{-5} mol·l^{-1} are given in Fig. 5. Adaptation of *Euglena* cells to a 50-fold increase in the external calcium concentration increases either the electrochemical force (arrow A, Fig. 5), and consequently the specific activity of the calcium pump, or the cytoplasmic free calcium (arrow B, Fig. 5). Whatever the case may be, the 10-fold increase in the total cellular calcium pool (from 1.6 mM to 15.1 mM) cannot be explained. Since the quantity of free cytoplasmic calcium represents at the very most 0.1% of the *Euglena* cell calcium, then the other calcium pools in the cell (bound calcium and stored calcium) must have increased. Considering the different stored calcium pools inside the cell, there is a general agreement that the nucleus, mitochondria and endoplasmic reticulum together contain more than 90% of the cell calcium, the rest being bound to molecules such as nucleic acids, phospholipids, ATP, amino acids, citrate, etc [23–26].

An intracellular potential difference was measured when transcellular electrical profiles were recorded. The reproductibility of the measurements, the sensitivity of the measured potential differences to calcium (Fig. 3), and the measured increase in resistance all indicate that these measurements represented transmembrane potentials.

The comparison of the potential versus the external calcium concentration in the plasma membrane and the impaled compartment membrane suggests that the impaired compartment accumulates calcium. The only noticable difference is the value of the so-called Nernst slope: 15 mV for the plasma membrane but only 6 mV for the impaled compartment membrane. This indicates in the latter case an increased difference between calcium activity and calcium concentration and suggests that calcium sequestered in this compartment for the most part is bound.

Since the measured resistance suggests a double-membrane enclosed compartment, the impaled calcium store-compartment may be either the mitochondrial network shown in these etiolated *Euglena* cells near the plasma membrane [27–28] or the nucleus. Previous results indicate that calcium is maintained low in the nucleus when external calcium concentration is increased [20]. The measured membrane potential difference (from +30 to +50 mV) is not consistent with most of the mitochondrial potentials registered (from −150 to −200 mV) using ionic probes or dyes [29–30] although positive mitochondrial membrane potentials (about +20 mV) have been previously reported in microelectrode studies [31–34]. The difference between measurements registered with microelectrodes and ionic probes

may come from the binding of these probes to membranes, mimicking an accumulation of ions and simulating the existence of a negative transmembrane potential. Such an artifact has already been demonstrated in isolated plant vacuoles [35] but can hardly explain a discrepancy between +20 mV and −150 mV; it would mean an over 1000-fold accumulation error of the probes. Nevertheless, if positive membrane potentials indeed do exist across the mitochondrial membrane, the Mitchell hypothesis would be contradicted. From our results, and in view of the fact that the results of the Tedeshi group [31–34] have not been reproduced, the nature of the compartment impaled by the microelectrode remains uncertain.

Whatever the nature of the impaled compartment, calcium accumulates inside *Euglena* cells, and among the endoplasmic reticulum and mitochondria, which are the two main calcium stores in the cell [26,36], the endoplasmic reticulum is involved in sequestering calcium at slow calcium injection, whereas mitochondria become active when the injection speed exceeds $20 \cdot 10^{-5}$ $mol \cdot s^{-1}$ [37]. Thus, Ca^{2+} homeostasis is primarily maintained by the endoplasmic reticulum, while mitochondria are involved only in case of 'emergency'. Since we can consider a 100 mM external calcium concentration an emergency case for the *Euglena* cells (external calcium concentration superior to 200 mM kills cells, Tamponnet, unpublished results), the mitochondrial network seems to be involved in the calcium accumulation of cells adapted to high external calcium concentrations even if it cannot be the impaled compartment.

In conclusion, K^+ and Cl^- internal concentrations, and the total internal Ca^{2+} pool have been determined in *Euglena gracilis* cells. The finding of a mean plasma membrane-potential difference of −58 mV, in accord with previous results on other microorganisms, suggests that this organism extrudes Ca^{2+} against the thermodynamic gradient. The changes in this potential difference and in the total internal calcium pool when cells are adapted to various external calcium concentration indicate that *Euglena* cells accumulate calcium when external calcium concentration increases. An internal compartment-membrane potential difference, positive inside, has been measured. These results suggest that this compartment accumulates calcium when *Euglena* cells adapt to increased external calcium concentration. From the previous results, the exact nature of this compartment is still unknown but mitochondria seem to be involved as a calcium store.

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