

Electrophysiology of Mycoplasma Membranes

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The influence of transmembrane ion fluxes on mycoplasma membrane potentials was studied. Fluorescence intensity of potential-sensitive carbocyanine dyes was calibrated vs. electric membrane potential. Potassium and sodium ion diffusion potentials significantly contributed to mycoplasma membrane potential. Chloride ions were obviously freely permeable across mycoplasma membranes. Under growth conditions the mycoplasma membrane potential was estimated to be $\Delta\psi = -80$ mV.

INTRODUCTION

The electric properties of excitable, i.e., nerve and muscle cell, membranes are well known and current research topics of electrophysiology. Presumably because of experimental difficulties, much less is known about the electric characteristics of other eu- and procaryotic cell membranes [1,2].

In previous investigations, we have applied several techniques to determine the electric potentials [3-7] and electrochemical proton potentials [8,9] across mycoplasma membranes. The electric potential ψ is a physical quantity to describe an electric field. It may be used to determine the energy of a charged particle in an electric field. The potential difference $\Delta\psi$ of two points is the voltage between both points. A potential difference hinders or favors the dislocation of a charged particle.

Various kinds of electric potentials are associated with biological membranes [10]. According to location, one may distinguish between transmembrane and membrane surface potentials. Transmembrane potentials are due to an electric field penetrating the whole membrane. The membrane potential is defined as the potential difference, $\Delta\psi$, measured between two identical reference electrodes on opposite sides of a membrane. Membrane surface potentials are located at the boundary between the membrane surface and the surrounding medium. From an energetic aspect, the electric membrane potentials may be divided into equilibrium and non-equilibrium potentials. Equilibrium potentials are obtained if a non-permeant ion is unequally distributed between two solutions separated by a membrane which is selectively permeable to certain ions. In Donnan equilibrium, the difference of electrochemical potentials of each ion on both sides of the membrane disappears. The Donnan potential corresponds to $\Delta\psi_{\text{Donnan}} = -(RT/F) \ln r$, where R is the gas constant; T, the absolute temperature; F, Faraday's constant; and r, the Donnan coefficient, $r = [\text{cation}]_{\text{int}}/[\text{cation}]_{\text{ext}} = [\text{anion}]_{\text{ext}}/[\text{anion}]_{\text{int}}$. Non-equilibrium or membrane diffusion potentials obtain whenever ions diffuse through a membrane down their con-

centration gradients. The mathematical treatment of membrane diffusion potentials is based on the general flux equations of Nernst. Systems with several electrolytes were studied by Planck. However, the quantitative treatment is complicated. Two major attempts were made to simplify Planck's equation and solutions. Goldman, Hodgkin, and Katz treated the electric potential gradient as linear. For the biologically most significant ions, their equations reads $\Delta\psi = -(RT/F) \ln \{(P_K + [K^+]_{int} + P_{Na} \cdot [Na^+]_{int} + P_{Cl} \cdot [Cl^-]_{ext} + A) / (P_K \cdot [K^+]_{ext} + P_{Na} \cdot [Na^+]_{ext} + P_{Cl} \cdot [Cl^-]_{int} + B)\}$; where P denotes the permeability coefficient of the corresponding ion; $[]_{ext}$ and $[]_{int}$, the extracellular and intracellular ion concentrations; A and B hold for the correspondent fixed charges. A different approach was tried by Henderson, who treated the concentration gradient within the membrane as linear. For most biomembranes, the Goldman-Hodgkin-Katz equation gives more realistic results. Obviously their hypothesis is more appropriate, since biomembranes are inhomogeneous in the direction perpendicular to the surface because the lipid core is a barrier with a high activation energy for ions. They are also inhomogeneous in the direction parallel to the surface due to the large amounts of integral and peripheral membrane proteins.

In this paper we present the data of our studies to estimate the influence of transmembrane ion fluxes on mycoplasma membrane potentials.

MATERIALS AND METHODS

The potential-sensitive fluorescent dye [11], 3,3'-dipropyl-2,2'-thiodicarbocyanine iodide [diS-C₃-5] was a generous gift of Dr. A. Waggoner, Amherst, MA, USA. Valinomycin was from Sigma.

Mycoplasma mycoides subsp. *capri* was grown in suspension at 37°C [8]. The pH was kept constant at 7.4 by automatic titration. Mycoplasmas were harvested immediately before fluorescence measurements by centrifugation at 8,000 g for 1.5 minutes at 24°C. For several experiments mycoplasmas were centrifuged at 12,000 g for ten minutes at 4°C and stored at 0°C for at least one hour.

The media used for determination of potassium diffusion potentials, contained 2 mM KCl + 270 (1 - 2⁻⁷) mM sucrose + 5 mM Tris, at pH 7.4, with $n = 1, 2, \dots, 6$, whereas in media used for measurement of sodium diffusion potentials, KCl was replaced by NaCl. For fluorescence measurements, the mycoplasmas were suspended in 3 ml of medium, and the optical density of the cell suspension was adjusted. The fluorescent dye was dissolved in ethanol, and 1 μ l was added to the mycoplasma suspension. The final concentration of the dye was 0.5 μ M. The fluorescence signal was recorded with a Hitachi-Perkin-Elmer fluorescence spectrophotometer 204 with the excitation wavelength set at 625 nm and the emission at 660 nm. When I_0 had been measured, valinomycin dissolved in ethanol was added directly to the cell suspension. The final concentration of valinomycin was 1.17 μ M.

Cation concentrations were measured by atomic absorption spectrophotometry, as described previously [4,6,7].

RESULTS AND DISCUSSION

Membrane potentials were determined by using potential-sensitive fluorescent carbocyanine dyes which distribute between medium and cells according to the membrane potential [11]. Membrane hyperpolarization, inside more negative, due to, for example, potassium ion exit induced by the potassium-specific ionophore valinomycin [12], was accompanied by an uptake of dye molecules and a decrease in

fluorescence intensity by dimerization to non-fluorescent aggregates. Membrane depolarization, inside less negative, by potassium ion uptake, induced an extrusion of dye molecules, thus diminishing the number of dimers and increasing fluorescence intensity.

Determination of Mycoplasma Membrane Potentials by the Null-Point Method

When we followed the null-point method [3,4,6,7,9], the changes in fluorescence intensity were plotted vs. \log_2 of the external ion concentrations. The resulting curve crossed the abscissa at the null-point, i.e., the critical external potassium ion concentration where potassium ions are in a Donnan equilibrium. The membrane potential could be calculated from the critical potassium ion concentration and the intracellular potassium concentration, as determined by atomic absorption spectrophotometry [4,6,7].

However, the values obtained by this method were valid only for the special ionic composition of the buffer solutions at the null point. The influences of ion diffusion could not be observed. In order to overcome these limitations, we now calibrated the fluorescence intensity vs. electric membrane potential. All experiments were done with *M. mycoides* subsp. *capri*.

Calibration of Fluorescence Intensity vs. Electric Membrane Potential

As indicated by a large decrease in fluorescence intensity (Fig. 1) immediately after addition of the dye to the mycoplasma suspension, the dye distributed between medium and cells according to the membrane potential. I_0 was taken at the end of the distribution, usually after about one to four minutes. Then valinomycin was added and caused an abrupt change in fluorescence intensity, due to an increase of potassium diffusion [12]. Maximum diffusion was obtained a few minutes after addition of valinomycin. At this time I_{val} was taken. I_{val} remained constant for different mycoplasma preparations even when the density of the cell suspension changed about ± 20 percent. Mycoplasmas suspended in media containing different sodium but constant potassium ion concentrations had different values of I_0 , but constant

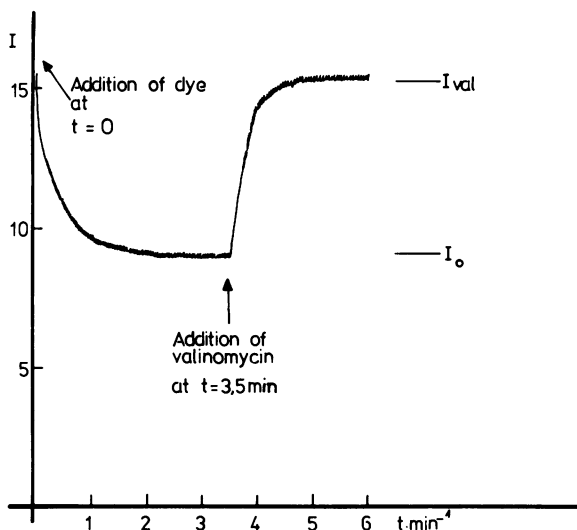


FIG. 1. Changes in fluorescence intensity vs. time obtained for *Mycoplasma mycoides* subsp. *capri* in 64 mM KCl + 202.5 mM sucrose + 5 mM Tris, pH 7.4, after addition of diS-C₃-(5) (final concentration, 0.5 μ M) at $t = 0$, and after the addition of valinomycin (final concentration, 1.17 μ M) at $t = 3.5$ minutes.

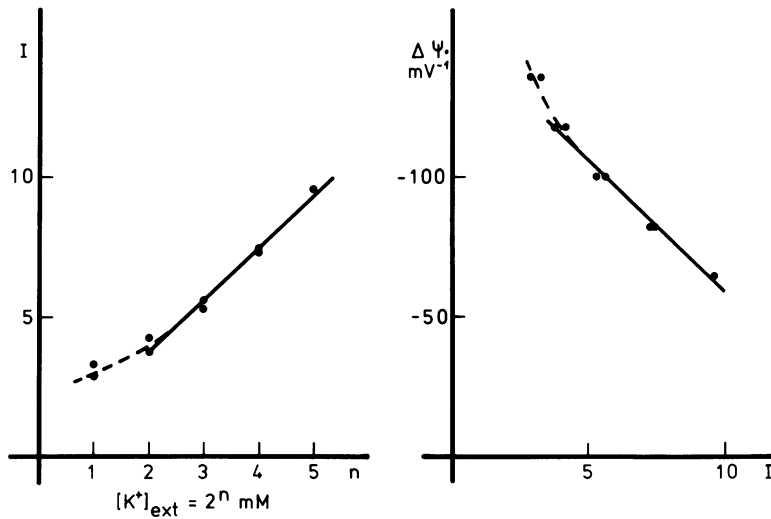


FIG. 2. Calibration curves: (*left*) Fluorescence intensity I (arbitrary units) plotted vs. \log_2 of external potassium ion concentration. Fluorescence intensity was measured after addition of valinomycin (final concentration, $1.17 \mu\text{M}$) to a suspension of *Mycoplasma mycoides* subsp. *capri* in 2^n mM KCl + $270(1 - 2^{n-7})$ mM sucrose + 5 mM Tris, pH 7.4, + diS-C₃-(5) (final concentration, $0.5 \mu\text{M}$) (*right*) $\Delta\psi$ plotted vs. fluorescence intensity. $\Delta\psi$ was calculated from $\Delta\psi = - (RT/F) \ln (400 \text{ mM} / [K^+]_{ext})$.

I_{val} . This clearly proved that, upon addition of valinomycin, the membrane potential was entirely determined by potassium ion diffusion.

The fluorescence intensity was plotted versus \log_2 of external potassium ion concentration (Fig. 2). A perfectly linear relationship was found for potassium ion concentrations ranging from 4 mM to 64 mM. For lower and higher potassium ion concentrations, deviations occurred. Membrane potential was proportional to fluorescence intensity from -120 mV to -60 mV. The reproducibility was excellent. Nevertheless for each preparation of mycoplasmas a complete calibration run was performed.

Influence of Potassium Ions

When the mycoplasmas were kept in growth medium at 37°C , and harvested by centrifugation for 1.5 minutes at 24°C , immediately before potential determination, the membrane potential proved to be strongly dependent upon external potassium ion concentration (Fig. 3a). The membrane potential increased nearly linearly from -120 mV to -85 mV when the external potassium ion concentration rose from 4 mM to 64 mM. Obviously a potassium ion diffusion potential significantly contributed to the mycoplasma membrane potential.

When the mycoplasmas were kept in growth medium at 37°C , harvested by centrifugation in the cold, and stored at 0°C for at least one hour, quite different values of the membrane potential were obtained; the membrane potential was about -135 mV and was nearly independent of the external potassium ion concentration, but responded to changes of external proton concentration (Fig. 3b). Membrane hyperpolarization was obviously due to positive ions, most probably protons, which had been metabolically generated, but could not be released in the cold. Upon suspen-

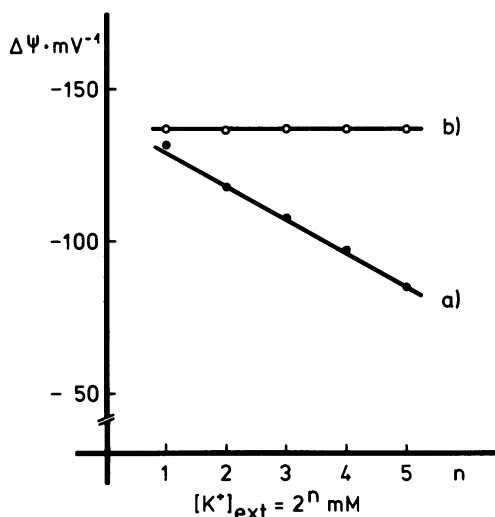


FIG. 3. Influence of potassium ions on mycoplasma membrane potential. Membrane potential of *Mycoplasma mycoides* subsp. *capri* plotted vs. \log_2 of external potassium ion concentration. a. Mycoplasmas harvested at 24°C immediately before fluorescence measurements. Membrane potential is linearly dependent upon $[K^+]_{ext}$. b. Mycoplasmas harvested at 4°C stored at 0°C for one hour. Membrane potential is independent of $[K^+]_{ext}$.

sion of cold mycoplasmas in incubation medium at 24°C, a rapid and massive proton diffusion occurred which determined the membrane potential. However, when cold mycoplasmas were again kept at 24°C for one hour before measurement, this effect vanished since protons reached equilibrium, and membrane potential was again dependent upon external potassium ion concentration.

Influence of Sodium Ions

The contribution of sodium ions to the mycoplasma membrane potential was determined by experiments analogous to those for potassium ions. The effects of sodium ions were measured with both freshly harvested mycoplasmas and organisms which had been harvested and kept in the cold. The results were quite similar to those with potassium ions, i.e., sodium ion diffusion significantly contributed to mycoplasma membrane potential.

Influence of Chloride Ions

Generally the negatively charged chloride ions are assumed to penetrate cell membranes readily. If this holds true for mycoplasmas, chloride ions should be in Donnan equilibrium and should not contribute to mycoplasma membrane potential. If, on the other hand, chloride ions are not in Donnan equilibrium, a change in membrane potential should be expected when chloride is replaced by a non-permeant, negatively charged ion, e.g., by sulfate. When the mycoplasmas were suspended in media containing potassium chloride or sulphate, respectively, no significant differences of membrane potentials were observed, thus indicating that chloride ions were indeed obviously freely permeable through mycoplasma membranes.

Estimation of Mycoplasma Membrane Potential under Growth Conditions

Because of the principal experimental difficulties, e.g., binding and distribution of the fluorescent probe to plasma lipoproteins in growth medium, direct determination of membrane potential of growing mycoplasmas was impossible. In order to estimate the magnitude of mycoplasma membrane potential under growth conditions, the following considerations were performed. We had found that the intracellular water volume of mycoplasmas suspended in isotonic sucrose solution

containing potassium ions was lower than that determined for mycoplasmas suspended in isotonic sodium chloride solution containing potassium ions. At present, we cannot definitely decide whether shrinking or swelling occurred in media containing sucrose or sodium ions, respectively, as compared to growth medium. The intracellular potassium ion concentration of growing mycoplasmas was estimated to be in the range between 220 mM and 410 mM. These limiting values were obtained in isotonic saline and sucrose solutions, respectively. This correlated with a possible deviation of the true membrane potential of growing mycoplasmas from those measured under our experimental conditions, of less than -15 mV. Indeed, when we simulated the extracellular concentrations of the biologically most significant ions, as derived from the composition of mycoplasma growth medium (i.e., sodium ions at 64 mM and potassium ions at 8 mM in isotonic sucrose), the mycoplasma membrane potential was measured to be $\Delta\psi = -80$ mV.

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