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Transmembrane proton-motive potential of *Spiroplasma floricola*

Ulrich Schummer and Hans Gerd Schiefer

Institut für Medizinische Mikrobiologie, Klinikum der Justus Liebig Universität, Schubertstr. 1, D-6300 Giessen, FRG

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In *Spiroplasma floricola*, the transmembrane proton-motive potential Δp was studied. It is composed of a transmembrane electric potential difference, $\Delta\psi$, and a transmembrane proton gradient, ΔpH, according to $\Delta p = \Delta\psi - (Z \cdot \Delta\text{pH})$. Using a potential-sensitive carbocyanine dye and 5,5′-dimethyl[2-^{14}C]oxazolidine-2,4-dione as probes, $\Delta\psi$ and ΔpH were measured at different $[H^+]$ of the medium, and Δp was calculated to be remarkably constant at −123 mV ± 16% over a wide range of external pH values. Inhibition experiments indicated that it is generated by a membrane-bound, electrogenic, proton-translocating ATPase.

Membrane potential; Proton gradient; Proton-motive potential; (Spiroplasma floricola)

1. INTRODUCTION

Spiroplasmas are helical, motile mycoplasmas [1,2] and rather delicate microorganisms. The factors determining their shape, and the driving forces of their rotatory, flexional and translational motility are unknown. A transmembrane proton-motive potential Δp has often been hypothesized to be essentially involved in microbial motility [3,4]. It is composed of a transmembrane potential $\Delta\psi$ and a transmembrane proton gradient ΔpH according to

$$\Delta p = \Delta\psi - [2.3 \cdot R \cdot T \cdot \Delta\text{pH}]/F = \Delta\psi - (Z \cdot \Delta\text{pH}),$$

where F is the Faraday constant; R, the gas constant; T, the thermodynamic temperature; and $Z = 2.3 \cdot R \cdot T/F$ (59 mV at $T = 310$ K) [5]. This study was initiated to measure the parameters, and to calculate the magnitude of the spiroplasmal transmembrane proton-motive potential which is assumed to contribute to the shape and motility of these microorganisms.

Correspondence address: U. Schummer, Institut für Medizinische Mikrobiologie, Klinikum der Justus Liebig Universität, Schubertstr. 1, D-6300 Giessen, FRG

The membrane potential $\Delta\psi$ was determined using a potential-sensitive fluorescent carbocyanine dye [6–9]. With this method the membrane potential is compared with a K^+ diffusion potential induced by addition of the K^+-specific ionophore valinomycin. External $[K^+]$ and buffer compositions are varied until, after addition of valinomycin, no changes in fluorescence intensity or internal $[K^+]$ occur thus indicating that $[K^+]$ is in Donnan equilibrium. At this very special $[K^+]$ (the 'null point' [7]; 'critical K^+ concentration' $\{[K^+]_{crit}\}$ [10,11]) the membrane potential equals the K^+ diffusion potential induced by valinomycin, and is calculated according to

$$\Delta\psi = (RT/F) \cdot \log\{[K^+]_{crit}/[K^+]_{int}\}.$$

The transmembrane proton gradient ΔpH was measured from the distribution of a weak organic acid between the intra- and extracellular water space [12,13].

2. MATERIALS AND METHODS

The fluorescent dye, 3,3′-dipropyl-2,2′-thiodicarbocyanine iodide [diS-C_3-(5)], was a generous gift from A. Waggoner, Amherst, now at the

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Carnegie-Mellon University, Pittsburgh, USA [6]. Valinomycin and carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) were from Sigma; *N,N'*-dicyclohexylcarbodiimide (DCCD) from Merck; 5,5'-dimethyl-[2-^{14}C]oxazolidine-2,4-dione (DMO) from Amersham, Buchler.

Spiroplasma floricola BNR1 [14] (kindly provided by J.C. Vignault and J.M. Bové, Pont-de-la-Maye, France) was grown in Hepes-buffered DSM 4 medium which contained per 100 ml, 1.5 g PPLO broth base (Difco), 8 g sucrose (Merck), 2.5 mg phenol red, 1.32 g Hepes, pH 7.5, and 10 ml inactivated (30 min at 56°C) horse serum (Flow). 500 mg glucose served as the nutritional substrate. Spiroplasmas were grown at 34°C with the pH kept constant at 7.5 by automatic titration. Shape and motility of the spiroplasmas were regularly monitored by dark-field microscopy. Spiroplasmas were harvested by centrifugation, resuspended in 1/10th the original volume of fresh medium, and further incubated at 25°C for 1 h with the pH kept constant at 7.5.

2.1. *Determination of* $\Delta\psi$

Isotonic media were prepared containing 2^n mM KCl + ($2^7 - 2^n$) mM NaCl + 5 mM Hepes, pH 7.5, with $n = 7, 6, 5, \ldots, 0$. A series of experiments was performed with the pH of the suspension medium varied between 8.5 and 5.5. Spiroplasmas were harvested immediately before fluorescence measurements by centrifugation at 8000 × *g* for 1 min in an Eppendorf 3200 bench centrifuge. The sedimented spiroplasmas were homogeneously resuspended in 3 ml of the medium samples to an absorbance at 509 nm of about 0.4 corresponding to a final protein concentration of 30 μg/ml. 1 μl diS-C_3-(5) dissolved in ethanol was added (final concentration, 0.5 μM). When the signal was constant, i.e., after about 1 min, the fluorescence intensity I_o was measured with a Hitachi Perkin-Elmer fluorescence spectrophotometer 204 with the excitation wavelength set at 625 nm and the emission at 660 nm. Then 1 μl valinomycin dissolved in ethanol was added (final concentration, 2.5 μM), and the fluorescence intensity I_{val} was recorded. Ethanol alone at a concentration of 0.67‰ did not change the fluorescence intensity.

Intracellular K^+ concentrations, $[K^+]_{int}$, were measured by atomic absorption spectrophotometry after digesting a weighed portion of the spiroplasmas by sonication in 0.5 ml of 1 M HCl [10,11].

2.2. *Determination of* ΔpH

After centrifugation the sedimented spiroplasmas were finally suspended in 1/10 of the original volume of the isotonic Hepes-buffered sucrose solution. Initially no nutrient substrates were added. The pH was kept constant at 8.0 by automatic titration. In one series the initial pH was 8.5. [^{14}C]DMO (final concentration, 0.1 μM) dissolved in saline was added. After taking a first sample, glucose (final concentration, 50 mg/ml) was added and served as the energy source. The extracellular pH determined with a glass electrode was allowed to drop by glycolysis. Samples were taken at pH (8.5), 8, 7.5, 7, 6.5, 6, 5.5 and 5, and harvested by centrifugation. The extra- and intracellular water volumes of the pellets were determined as described in [10,11]. The radioactivity in the cell pellets and in 100 μl of the supernatants was measured by scintillation spectrophotometry. ΔpH was calculated according to

$$\Delta pH = pH_{int} - pH_{ext}$$
$$= pK_{DMO} - pH_{ext} + \log\{[C_{int}/C_{ext}][10^{(pH_{ext} - pK_{DMO})} + 1] - 1\}$$

where pH_{int} and pH_{ext} are the intra- and extracellular pH; pK_{DMO}, the negative logarithm of the apparent dissociation constant of DMO, approx. 6.2 at 37°C; and C_{int} and C_{ext}, the intra- and extracellular concentrations of DMO [8,12,13].

In experiments with inhibitors, DCCD and CCCP dissolved in ethanol were added to the cell suspensions after the addition of DMO. The same amount of ethanol alone did not change ΔpH.

3. RESULTS AND DISCUSSION

3.1. *Determination of* $\Delta\psi$

A marked fluorescence signal, I_o, was obtained after the addition of the potential-sensitive fluorescent dye, diS-C_3-(5), to a suspension of *S. floricola* in Hepes-buffered saline. The intensity of fluorescence on the addition of valinomycin (I_{val}) was found to depend upon $[K^+]_{ext}$, i.e., increasing $[K^+]_{ext}$ by successively substituting KCl for NaCl in the suspension medium led to a smaller decrease or even increase in fluorescence intensity on the addi-

tion of valinomycin. When $\Delta I/I_o$ ($\Delta I = I_o - I_{val}$) was plotted versus the $\log_2$ of the corresponding $[K^+]_{ext}$ (fig.1), the curve obtained had a sigmoid shape. This has been derived theoretically and discussed in detail elsewhere [15]. However, within a considerable range around the center of symmetry, it can be approximated by a straight line which crosses the abscissa at $n = 3.7$, corresponding to $[K^+]_{crit} = 13$ mM. At this point $[K^+]$ is in Donnan equilibrium. From the values of the spiroplasmal $[K^+]_{int}$ determined by atomic absorption spectrophotometry, and $[K^+]_{crit}$, the membrane potential $\Delta\psi$ at pH 7.5 was calculated to be -80 mV (table 1). When the pH of the suspension medium was varied between pH 5.5 and pH 8.5, $\Delta\psi$ varied between -55 mV and -100 mV, respectively (fig.3).

Table 1

Physical and chemical data compiled for the calculation of *S. floricola* membrane potential

dw	0.198 ww
V_t	0.802 ww
V_{ext}	0.494 ww
V_{int}	0.308 ww
$[K^+]_{crit}$	13 mM
$[K^+]_{int}$	300 mM
$\Delta\psi$	-80 mV

dw, dry weight; ww, wet weight; V_t, total pellet water volume = wet weight minus dry weight; V_{ext}, extracellular water volume; V_{int}, intracellular water volume = $V_t - V_{ext}$; $[K^+]_{crit}$, critical external $[K^+]$ for which no changes in fluorescence were observed after the addition of valinomycin; $[K^+]_{int}$, intracellular potassium concentration; $\Delta\psi$, membrane potential

3.2. *Determination of ΔpH*

Starving spiroplasmas produced only minimal amounts of protons and were unable to maintain a transmembrane proton gradient, i.e., ΔpH approximated zero. A few minutes after the addition of glucose the spiroplasmas started to eject protons into the suspension medium. The intracellular pH of the metabolizing spiroplasmas was found to remain more alkaline than the extracellular medium. For example, at an extracellular pH of 7, the intracellular pH was 7.54. When the external pH decreased from 8 to 5 by the metabolic activity of the spiroplasmas, the transmembrane proton gradient ΔpH increased from near 0.5 to about 1.8, while the intracellular pH decreased from 8.53 to about 7 (at $pH_{ext} = 6$), and then remained constant. Our results are summarized in fig.2 where the intracellular pH of metabolizing spiroplasmas was plotted versus the extracellular pH in the medium.

Inhibitors of the transmembrane proton gradient were tested over a wide range of concentra-

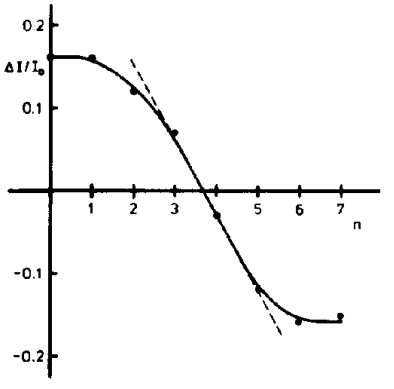

Fig.1. Changes in fluorescence intensity $\Delta I/I_o$ [$\Delta I = I_o - I_{val}$] of diS-C_3-(5) in a suspension of *S. floricola* plotted versus $\log_2$ of the corresponding external potassium concentrations. The intersection point of the curve with the abscissa at $n = 3.7$ indicates $[K^+]_{crit} = 13$ mM.

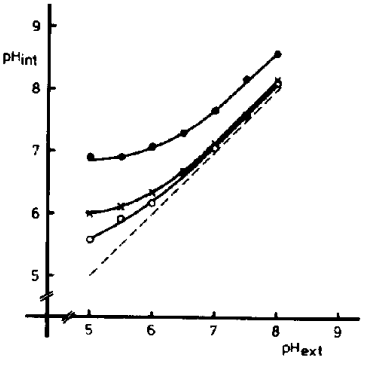

Fig.2. The intracellular pH of glycolyzing *S. floricola* plotted versus the extracellular pH (●); in the presence of DCCD, final concentration, 25 μg/ml (×); in the presence of CCCP, final concentration, 12.5 μg/ml (○). The dotted line indicates the 'iso-pH curve', i.e. $pH_{int} = pH_{ext}$.

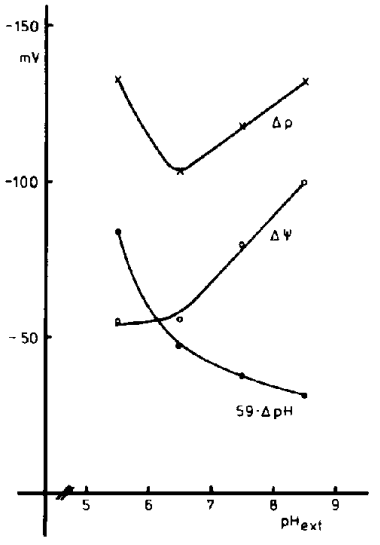

Fig.3. Effect of medium pH on the transmembrane proton-motive potential Δp and its parameters $\Delta\psi$ and ΔpH.

tions. Dose-dependent effects were found. The results diagrammed in fig.2 were obtained with the smallest possible concentrations of the substances sufficient to give maximum inhibitory effects. ΔpH was strongly influenced or even dissipated by DCCD which is an inhibitor of membrane-bound ATPase, and CCCP which is a proton conducting uncoupler [4,16].

3.3. *Calculation of the transmembrane proton-motive potential* Δp

Δp was calculated according to $\Delta p = \Delta\psi - (Z \cdot \Delta\text{pH})$. As shown in fig.3, $\Delta\psi$ strongly depended upon pH_{ext}, and decreased from -55 mV (at $pH_{ext} = 5.5$) to -100 mV (at $pH_{ext} = 8.5$). ΔpH was found to depend also upon pH_{ext}, but in the opposite direction, i.e., ΔpH increased from -84 mV (at $pH_{ext} = 5.5$) to -31 mV (at $pH_{ext} = 8.5$). Δp was calculated to be $\Delta p = -131$ mV (at $pH_{ext} = 8.5$); $\Delta p = -118$ mV ($pH_{ext} = 7.5$); $\Delta p = -103$ mV (at $pH_{ext} = 6.5$); and $\Delta p = -139$ mV (at $pH_{ext} = 5.5$). We conclude that Δp is kept remarkably constant at $\Delta p = -123$ mV $\pm$ 16% over a wide range of pH_{ext} values. Its magnitude is distinctly higher than that of other mycoplasmal species [7–9].

The inhibition experiments indicate that the transmembrane proton-motive potential of *S. floricola* is generated by a membrane-bound, electrogenic, proton-translocating ATPase which operates in the direction of hydrolysis of ATP formed by glycolysis, and leads to proton extrusion.

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