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ACTIVE K^+ TRANSPORT IN *MYCOPLASMA MYCOIDES* VAR. CAPRI

RELATIONSHIPS BETWEEN K^+ DISTRIBUTION, ELECTRICAL POTENTIAL AND ATPase ACTIVITY

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Summary

The addition of $5 \cdot 10^{-5}$ M or less of dicyclohexylcarbodiimide to *Mycoplasma mycoides* var. Capri preferentially influences K^+ influx rather than efflux and reduces by 30—40% the activity of the membrane-bound Mg^{2+}-ATPase. Adding valinomycin to metabolizing cells does not markedly affect K^+ distribution but induces a rapid and complete loss of intracellular K^+ in non-metabolizing cells. Uncoupling agents such as dinitrophenol, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, dissipate the K^+ concentration gradient only when combined with valinomycin.

Variations in the merocyanine fluorescence intensity indicate that a transmembrane electrical potential ($\Delta\psi$) is generated on cell energization. This $\Delta\psi$, not affected by valinomycin or uncouplers when used alone, is collapsed by a mixture of both. No change in fluorescence intensity can be detected when glucose is added to dicyclohexylcarbodiimide treated organisms.

These experiments suggest that the membrane-bound Mg-ATPase activity controls K^+ distribution in these organisms through the generation of a transmembrane electrical potential difference.

Abbreviations: DCCD, dicyclohexylcarbodiimide; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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Introduction

Observations reported in a previous paper [1] indicate that *Mycoplasma mycoides* var. Capri (PG3) actively accumulate K^+ against a high concentration gradient. The failure of ouabain to alter the K^+ accumulation process suggests that cell cation content is not controlled by a Na^+-K^+-activated ATPase. On the other hand, dicyclohexylcarbodiimide (DCCD) was shown to block the K^+ influx which takes place in freshly harvested organisms metabolizing glucose (hereafter referred to as energized cells); in addition, once depleted of their internal K^+, the K^+-depleted organisms poisoned with DCCD were no longer able to reaccumulate K^+ when sugar was added to the cell suspension.

Taking into account previous conclusions derived from the analysis of DCCD effects on the transport properties in other microorganisms [2—4], the aforementioned observations might indicate that the membrane-bound Mg^{2+}-dependent ATPase activity and, more generally the chemiosmotic phenomenon [5—7], plays a determinant part in energy conservation and tranduction in mycoplasma. Indeed, in bacterial systems under anaerobic conditions, or where respiratory activity is either defective or lacking, DCCD causes a drastic reduction in the transport of K^+ and other solutes [8—10]. Some workers in this field [2—4] have explained these observations in terms of Mitchell's chemiosmotic concept. Briefly, it is thought that, by specific interaction with the membrane-bound Mg^{2+}-dependent ATPase, DCCD abolishes the electrochemical potential gradient for protons ($\Delta \mu H$) generated across the cell membrane by the ATPase. This in turn, depresses the transport and accumulation of solutes. Recent evidence shows that in respiring bacteria and derived membrane vesicules, the $\Delta \mu H$ generated by the respiratory chain plays a similar role [11—13].

Since a Mg^{2+}-dependent ATPase activity has been reported in the mycoplasma membrane [14,15], we have attempted to determine if the active K^+ transport mechanism of PG3 could also be explained in terms of the chemiosmotic hypothesis. The present paper describes the effects of valinomycin, uncouplers and DCCD on the K^+ transport parameters, the Mg^{2+}-ATPase activity and the transmembrane potential of the PG3 strain. Changes in $\Delta\psi$ were estimated from the variations in the fluorescence intensity of merocyanine 540. A brief account of the results has already been published [16].

Methods

The growth and collection of PG3 were carried out as per methods described in a previous paper [1]. The effects of different chemical agents on intracellular K^+ content were analyzed on washed cells (0.2 mg/ml) resuspended in a buffered saline solution (100 mM sodium phosphate, 35 mM NaCl; 2 mM $MgCl_2$; 1 mM KCl; pH 7.2 at 37° C). In experiments using K^+-depleted cells, intracellular K^+ content was reduced by incubating freshly harvested organisms in glucose-free medium for 2 h at 37° C. K^+ content was measured as per description [1].

pH measurements

Essentially, the method described by Mitchell and Moyle [17] was followed,

i.e., cells (2—3 mg/ml) were resuspended in a slightly buffered solution (2 mM sodium phosphate; 150 mM NaCl; 2 mM $MgCl_2$) in a temperature-controlled cuvette (37° C). The variations in the cell suspension pH that followed the addition of small amounts of HCl were measured with a glass electrode and recorded.

Membrane preparation and determination of ATP activity

Membranes were isolated according to Rottem et al. [15]. The procedure described by Van Golde et al. [18] was also used in some experiments. Isolated membranes were resuspended in a diluted (1/20) buffer [19] and kept frozen (−45° C) until needed.

To determine ATPase activity, frozen membranes were thawed and diluted in a saline solution containing 5 μM $MgCl_2$, 50 μM Tris-HCl (pH 8.0) and 4.4 μM NaCl per ml. The reaction was started by adding 1.25 μM of unlabelled ATP or [γ-^{32}P]ATP, 0.5 μCi/ml, Amersham). When unlabelled ATP was used, the reaction was stopped after 20 min by adding 1 ml 10% cold trichloroacetic acid. After centrifugation at 3000 × *g* for 10 min, the liberated P_i in the supernatant was determined according to Chen et al. [20] and used to calculate the ATPase activity. When [γ-^{32}P]ATP was utilized, the Biais method [21] was followed: 0.15-ml samples were collected after various intervals of incubation with radioactive ATP (generally 5, 10 or 15 min). The reaction was stopped with 0.3 ml cold solution containing 25% activated charcoal (Sigma) in 5% trichloroacetic acid. After centrifugation, aliquots of the supernatant were collected and counted in vials containing Instagel. Liberated P_i was calculated from the known specific activity.

Fluorescence measurements

Fluorescence of merocyanine 540 (Eastman Kodak Co.) was measured with a Perkin Elmer Spectrofluorimeter MPF 44A equipped with a Hamamatsu R777 photomultiplier tube. The excitation wavelength was 536 nm and the emission was recorded at 581 nm, both with a 7 nm light band-pass. We filled 1 cm path-length quartz cuvettes with 2 ml phosphate buffer containing merocyanine ($5 \cdot 10^{-6}$ M) and about 250 μg/ml cells ($A_{640} \approx 0.35$). The cell suspension was continuously stirred with a magnetic stirrer and maintained at 37° C using a thermostatted cuvette holder.

Preliminary experiments were performed to check that the merocyanine concentration used in the fluorescence measurements did not affect growth or regulation of the K^+ content in organisms.

Chemicals

DCCD (Aldrich-Europe, Jansen Pharmaceutica N.V.), CCCP, FCCP (Boehringer, Mannheim) and valinomycin (Sigma) were prepared as concentrated ethanolic stock solutions to reduce the amount of alcohol added to the final cell suspension. Ouabain and 2-4 dinitrophenol were respectively obtained from Mann Research Lab Inc. and Aldrich-Europe.

Results

Effects of DCCD on K^+ cell content

It has been previously observed that DCCD ($5 \cdot 10^{-5}$ M) completely blocks

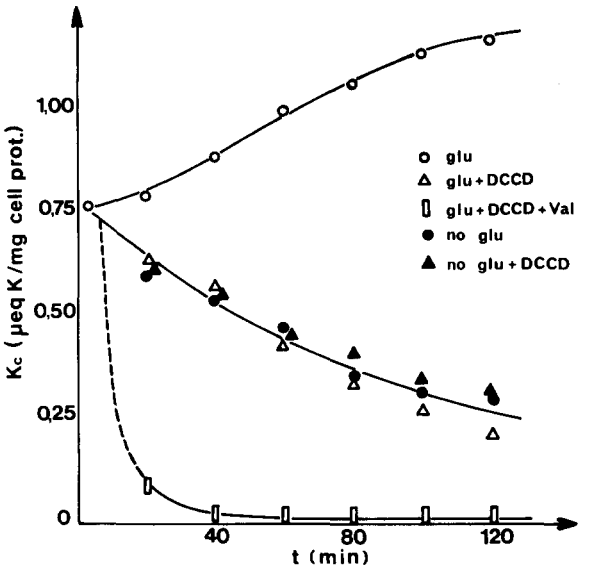

Fig. 1. Effect of DCCD on cell K^+ level of freshly harvested organisms. Washed organisms (0.2 mg/ml) were resuspended in 1 mM potassium phosphate buffer at 37°C. When present, glucose (20 mM) was added at the beginning of incubation; DCCD ($5 \cdot 10^{-5}$ M) and valinomycin (10^{-7} M) were added 5 and 10 minutes later. ●, no glucose; ○, glucose; △, glucose and DCCD; ▲, DCCD alone; □, glucose + DCCD + valinomycin. Ordinate: cell K^+ content (Kc) expressed as µequiv. K^+/mg protein.

the K^+ influx into freshly harvested organisms resuspended in media containing glucose. In these conditions, the poisoned cells are unable to maintain a high and steady intracellular K^+ level (Fig. 1). Thus, while glucose-energized cells slightly increase their K^+ content as a function of time, DCCD-treated organisms, although still able to metabolize the sugar, clearly lose their internal K^+. This K^+ loss rate is slow and comparable to that observed in untreated cells resuspended in a glucose-free medium. DCCD alone has no effect on the K^+ leak in non-metabolizing cells (Fig. 1). These observations therefore indicate that DCCD affects the K^+ entry process rather than the K^+ leakage, along the K^+ chemical concentration gradient.

The extent of K^+ influx inhibition by DCCD is concentration dependent. This is outstanding from experiments in which the DCCD dose-effect-relationship was studied on the K^+-reaccumulation process that takes place in cells previously depleted of their internal K^+ (Table I). This table shows that raising the DCCD concentration in K^+-depleted cell suspension progressively hampers the glucose-induced K^+ recovery. At $5 \cdot 10^{-5}$ M DCCD, K^+ reaccumulation is completely blocked; it should be added that in this situation, repeated attempts to detect transient K^+ uptake by tracer techniques failed. From Table I it is also apparent that higher carbodiimide concentrations produce secondary effects. Thus, cells incubated with 10^{-4} M DCCD and glucose have a lower K^+ content than that found in unpoisoned organisms which remain in a glucose-free medium, suggesting that DCCD increases K^+ efflux. A similar observation was reported by Harold [8] in *Streptococcus faecalis* and is probably related to the high reactivity of DCCD towards membrane components.

On the basis of these observations it can be concluded that keeping the con-

TABLE I

EFFECTS OF DCCD, FCCP AND VALINOMYCIN ON GLUCOSE-DEPENDENT K^+ REACCUMULATION IN K^+-DEPLETED ORGANISMS

Once the cellular K^+ level was reduced by 80—90% after 2 h incubation in a glucose-free buffer at 37° C, cells were equilibrated for 10 min with the desired inhibitor concentration: glucose (20 mM) was then added to the cell suspension and K^+ reaccumulation was estimated from the amount of K^+ uptake during the following 30 min. Results are expressed in relation to control conditions.

Inhibitor	Concn. (M)	Reaccumulation (%)
None	—	100
DCCD	10^{-6}	100
	10^{-5}	54
	$5 \cdot 10^{-5}$	3
	10^{-4}	−15
Valinomycin	$5 \cdot 10^{-7}$	86
FCCP	$5 \cdot 10^{-6}$	95
Valinomycin + FCCP		6

centration lower than $5 \cdot 10^{-5}$ M, DCCD preferencially acts on K^+ entry rather than efflux. In comparison with its mode of interaction with transport mechanisms in bacterial systems [2—4], this would suggest that DCCD blocks the driving force for K^+ transport ($\Delta \mu H$) via an inhibition of the Mg^{2+}-dependent ATPase activity.

Effect of DCCD on the membrane-bound Mg-dependent ATPase activity

The rate of ATP hydrolysis by isolated membrane suspension varied from 0.6 to 1.1 μM P_i/mg membrane protein/5 min depending on the membrane batch. These values compare well with those reported by Rottem et al. [15]. The ATPase activity, independent of the pH between 7.2 and 8.2, was neither stimulated by Na^+ and/or K^+ nor markedly influenced by the presence of ouabain (Table II). In contrast, a significant inhibition (30—40%) was observed in membranes preincubated for 10 min with $5 \cdot 10^{-5}$ M DCCD. Increasing the carbodiimide concentration to 10^{-4} M or the preincubation period did not affect the extent of inhibition. This indicates that although mycoplasma ATPase is more tightly bound to the membrane than microbial ATPase [22], its properties are very similar.

TABLE II

EFFECTS OF VARIOUS AGENTS ON MEMBRANE-BOUND Mg-DEPENDENT ATPase ACTIVITY

Isolated membranes were incubated for 10 min in the presence of each agent prior to ATPase activity determination. Results are expressed as % inhibition in relation to control conditions.

Compound	Concn. (M)	Inhibition (%)
DCCD	$5 \cdot 10^{-5}$	35
	10^{-4}	37
FCCP	$5 \cdot 10^{-6}$	7
Valinomycin	$5 \cdot 10^{-7}$	6
FCCP + valinomycin	$5 \cdot 10^{-6}$ & $5 \cdot 10^{-7}$	6
Ouabain	10^{-3}	4

The reduction in the rate of ATP hydrolysis produced by DCCD is clearly limited and consistently lower than values reported for bacterial membranes [8]. This might reflect the existence of ATP hydrolyzing enzymes different from the Mg-dependent ATPase, or a partial reorganization of membrane components produced by the repeated hypotonic washes during the isolation of membranes. Although it has been reported that the catalytic unit of the ATPase complex cannot be washed out of the membrane by these treatments [22], it could reduce the interaction between this component and the DCCD-reactive part. Such an interaction is essential to render the ATPase complex sensitive to DCCD [8].

Effects of uncoupling agents on cell K^+ content

If one accepts that K^+ transport in mycoplasma cells is driven by a $\Delta \mu H$ (or one of its components), one would expect proton conductors like dinitrophenol, FCCP or CCCP to be able, by short-circuiting $\Delta \mu H$ to dissipate the K^+ gradient across the membrane or to prevent K^+ reaccumulation [2—6].

Fig. 2 illustrates the effect of FCCP ($5 \cdot 10^{-6}$ M) on the cell K^+ content of freshly harvested organisms energized with glucose. Clearly, FCCP did not produce the expected K^+ gradient dissipation. Similar negative results were noted in the presence of higher FCCP concentrations (up to $5 \cdot 10^{-5}$ M) or when CCCP ($5 \cdot 10^{-6}$ M) or dinitrophenol (1 mM) were used.

The reaccumulation process triggered by energization with glucose in K^+-depleted cells was also insensitive to the three uncoupling agents (Table I), the maximal inhibition recorded with FCCP in three different experiments being 10%. No acceleration in the rate of K^+ loss from non-metabolizing cells was observed in the presence of the different uncoupling agents.

The experiment illustrated on Fig. 3 rules out the possibility that FCCP fails to alter the chemical K^+ gradient because it has no access to the core of the membrane. When HCl was added to a slightly buffered suspension of starved

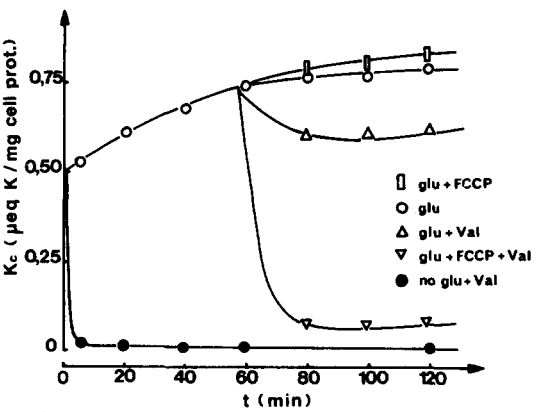

Fig. 2. Effects of FCCP and valinomycin on cell K^+ level of freshly harvested organisms. Experimental conditions as for Fig. 1. Open symbols: glucose was present at 20 mM; at 65 min, the following additions were made: □, FCCP ($5 \cdot 10^{-6}$ M); △, valinomycin (10^{-7} M); ▽, FCCP + valinomycin at the concentrations indicated above. Closed symbols: ●, cells resuspended in a glucose-free medium and treated at zero time with valinomycin.

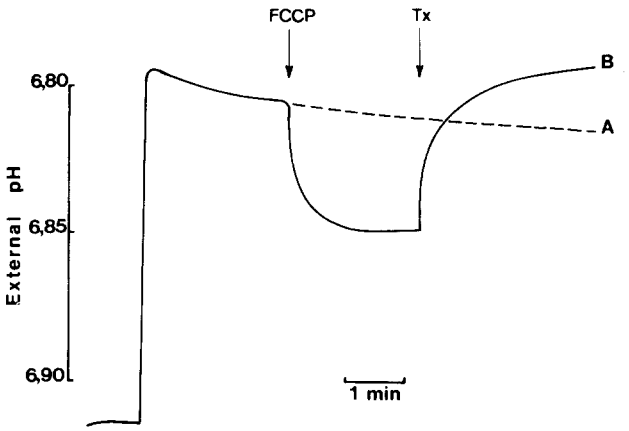

Fig. 3. Facilitation of transmembrane proton movements by FCCP. Curve A: time-dependent change in external pH that followed the addition of a small amount of HCl to a slightly buffered cell suspension. Curve B: Idem, but followed by the addition of FCCP ($5 \cdot 10^{-6}$ M) and then of triton (0.1%) as indicated by arrows.

organisms, there was an immediate rise in the medium pH followed by a slow return to a more alkaline value (trace A). This slow change in the external pH value has already been considered as evidence of proton backflux into the cells [17,23]. Trace B shows that FCCP addition greatly enhances the rate of this H^+ backflux, proving that FCCP effectively conducts H^+ across the mycoplasma membrane. Similarly, when a transient alkalinization of the pH medium is produced by NaOH, FCCP quickens the rate at which pH of the medium returns to the more acidic basal level, i.e., an H^+ efflux occurs.

Effects of valinomycin on K^+ content

The observation that microorganisms [24] or organelles from eukaryote cells [6] still accumulate K^+ in the presence of valinomycin is generally held to prove the existence of a transmembrane electrical potential. We therefore used this experimental approach to detect the existence of a $\Delta\psi$ in mycoplasma. Valinomycin was first tested for its effect on the cell K^+ level in freshly harvested organisms (Fig. 2). Addition of 10^{-7} M valinomycin to cells energized with glucose produced a limited drop in K^+ content compared to control conditions. The new lower steady value was reached within 10 min. In contrast, the addition of valinomycin to cells resuspended in glucose-free medium caused a complete release of intracellular K^+. This drop occurred at a rate much more rapid than the K^+ leak from control cells (comparison with data on Fig. 1). This K^+ release was not associated with any measurable change in cell volume but was compensated for at least 60%, by a gain in Na^+; a participation of protons in the cation exchange was suggested by the transient alkalinization observed in slightly buffered cell suspension when valinomycin was added (data not shown).

The limited effect of valinomycin on K^+ distribution across the membrane of cells energized with glucose can be modified by pretreating the organisms with either DCCP or FCCP: cells incubated with glucose for 5 min and then

poisoned with DCCD ($5 \cdot 10^{-5}$ M) during the following 10 min, completely lose their internal K^+ as soon as valinomycin is added (Fig. 1). The rate at which the K^+ concentration gradient dissipates is greater than the rate of K^+ loss in starved cells. It is comparable to that noted when valinomycin is added to non-metabolizing cells. The effect of valinomycin was also modified by pre-incubating cell metabolizing glucose with FCCP ($5 \cdot 10^{-6}$ M), which by itself did not drastically affect the K^+ distribution. As shown on Fig. 2, the addition of valinomycin to FCCP pretreated cells induced a complete and rapid drop in cell K^+.

Table I summarizes the observed effect of valinomycin, alone or in addition to FCCP, on the glucose-dependent K^+ reaccumulation which takes place in K^+-depleted cells. It can be observed that this net K^+ uptake mechanism is similarly insensitive to valinomycin and completely inhibited by the addition of a mixture of valinomycin and FCCP. In these latter experiments, again no transient $^{42}K^+$ influx was detected.

Neither valinomycin nor FCCP, used singly or in combination affected the rate of ATP hydrolysis by membrane preparations (Table II).

Electrical membrane potential and changes in merocyanine fluorescence

Several laboratories have reported that fluorescent dyes can effectively monitor the electrical membrane potential difference. This is particularly useful for microorganisms whose electrical properties cannot be studied by conventional microelectrode techniques because of the reduced cell size [25—27]. Fig. 4 summarizes the information obtained with such a technique on myco-

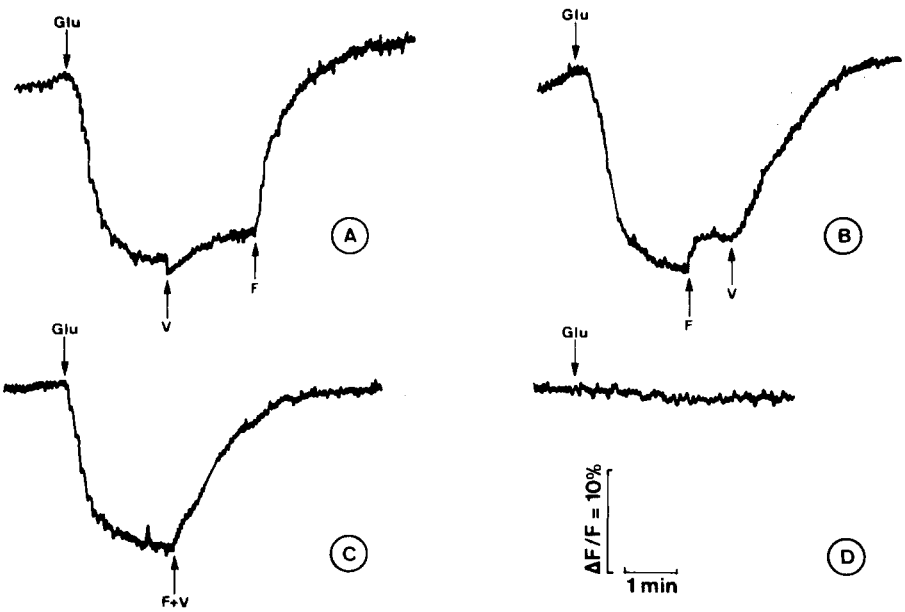

Fig. 4. Relative change in merocyanine fluorescence intensity ($\Delta F/F$). As indicated by arrows, glucose (Glu), valinomycin (V) and FCCP (F) were added to give final concentrations of 20 mM, 10^{-7} and $5 \cdot 10^{-6}$ M respectively. In conditions corresponding to trace D, cells were previously incubated for 10 min with DCCD ($5 \cdot 10^{-5}$ M) before glucose addition.

plasma cell suspension, using the merocyanine fluorescent probe.

When merocyanine ($5 \cdot 10^{-6}$ M) was added to a suspension of freshly harvested organisms, the dye fluorescence intensity increased and was accompanied by a 10 nm red shift of the maximum emission wavelength (572 → 582 nm). At 582 nm, a constant fluorescence level was generally reached within 2 min. This was sometimes followed by a time-dependent increase in the fluorescence intensity emission.

As shown in Fig. 4, the steady level of fluorescence intensity was markedly influenced by the addition of glucose: a rapid and large drop in fluorescence intensity took place within 1 min and represented 18—27% quenching of the initial fluorescence intensity. According to Waggoner [28], such a quenching reflects the fact that the cell interior becomes electrically more negative with respect to the outer bathing solution. No variation was noted when broken cells or membrane preparations were used, even when ATP was added instead of glucose. Finally, transient quenching of fluorescence intensity was observed when non energized cells, resuspended in K^+-free medium, were treated with valinomycin, a condition where electrogenic K^+ efflux takes place [29]. Neither valinomycin nor FCCP had any profound effect per se on the quenched signal recorded from energized cells. In contrast, addition of FCCP to valinomycin pretreated organisms or of valinomycin to FCCP poisoned cells, clearly produced a return of the fluorescence intensity to levels comparable to that measured before glucose addition. Simultaneous addition of both agents produced a similar reduction of the glucose induced quenching effect. Finally, the addition of glucose to organisms previously poisoned with DCCD ($5 \cdot 10^{-5}$ M), is no longer able to promote quenching of the fluorescence signal.

Discussion

The observations reported in this communication indicate that in PG3, K^+ distribution, ATPase activity and transmembrane electrical potential are interrelated.

First, the contribution of a membrane-bound Mg^{2+}-dependent ATPase to the K^+ regulation mechanism is suggested because both K^+ influx and ATPase activity are reduced in the presence of DCCD, a well known inhibitor of the ATPase activity [2—4].

It is to be stressed that the maximum inhibition of both ATPase activity (Table II) and K^+ uptake is observed for the same DCCD concentration ($5 \cdot 10^{-5}$ M). Since at this concentration no effect on K^+ efflux was noted, and because the rate of acid production consecutive to glucose metabolism is decreased by 40% only, we favor the hypothesis that K^+ influx inhibition is secondary to the reduction in ATPase activity.

Secondly, the use of valinomycin enables us to show that K^+ distribution is governed by an energy-dependent transmembrane $\Delta\psi$. Thus, adding valinomycin, which has the property to greatly increase the passive permeability of the membrane to K^+ [2,6], does not lead to a complete redistribution of K^+ across the membrane of metabolizing cells. It necessarily follows that the steady K^+ concentration gradient is compensated by a transmembrane $\Delta\psi$, the cytoplasmic side being electrically negative in relation to the outer solution so

that the electrochemical potential of K^+ is identical on both sides of the membrane [2,5,6]. In this particular condition, the magnitude of $\Delta\psi$ can be estimated from the K^+ distribution by using the Nernst equation [30—32]. Taking data derived from the experiment illustrated on Fig. 2 and a mean intracellular water volume of 4.8 μl/mg cell protein [1], $\Delta\psi$ is found to equal 125 mV. Values as high as 140 mV were computed from other experiments.

Extending such a calculation to valinomycin untreated cells, it appears that those organisms can develop a transmembrane $\Delta\psi$ as high as 150 mV, i.e., only from 10—25 mV higher than the value found in the presence of the antibiotic. Finally, the fast and complete release of cellular K^+ which follows valinomycin addition to cells resuspended in a glucose-free medium clearly contrasts with the limited effect of the K^+ ionophore on K^+ distribution in energized cells. This observation indicates that $\Delta\psi$ generation is under metabolic control.

The observed variations of merocyanine fluorescence intensity illustrated on Fig. 4 provide strong support of the hypothesis that a transmembrane $\Delta\psi$ is generated across the cell membrane as a result of glucose utilization. Thus, according to previous studies in which fluorescent dyes were used as indicators of transmembrane $\Delta\psi$ [25—28], a large quenching of merocyanine fluorescence intensity is expected to take place if a transmembrane $\Delta\psi$, negative inside the cell, is to be generated on cell energization [28]. The assumption that the drop in the dye fluorescence emission is related to a $\Delta\psi$ generation is supported by the finding that a transient fluorescence quenching is observed when starved cells, resuspended in a low K^+ (but not high K^+) medium, are treated with valinomycin. It has been shown [29] that the resulting K^+ efflux (Fig. 2) is electrogenic and as a consequence generates a transient $\Delta\psi$ which is negative inside the cell.

At this level of discussion, it appears that the energy-dependent K^+ distribution requires integrity of the membrane-bound Mg^{2+}-dependent ATPase activity and is governed by a transmembrane $\Delta\psi$. Two observations suggest that the ATPase contributes to the generation of $\Delta\psi$. First, fluorescence quenching is no longer observed in cells treated with the ATPase inhibitor DCCD (Fig. 4), suggesting that they are unable to generate a $\Delta\psi$. Moreover, these DCCD-treated cells, although resuspended in the presence of glucose, completely lose their internal K^+ after valinomycin addition (Fig. 1). This complete release of cell K^+ is simply interpreted by assuming that the K^+ chemical concentration gradient is no longer compensated by a transmembrane $\Delta\psi$, a situation prevailing in the absence of energy supply.

Our results suggest that the membrane-bound Mg^{2+}-dependent ATPase generates an electrical potential difference which in turn governs the distribution of K^+ across the membrane. This conclusion fits into the framework of Mitchell's chemiosmotic hypothesis [5—7]. Mitchell postulates that under anaerobic conditions or when microbial systems lack respiratory activity, the ATPase pumps protons outward electrogenically, building up an electrochemical potential difference for H^+ ($\Delta\mu H$) across the membrane. $\Delta\mu H$ is the sum of an electrical ($\Delta\psi$) and a chemical (Δ pH) component related by the following equation:

$$\Delta\mu H = \Delta\psi - 2.3\, RT/F\, \Delta \text{ pH}$$

Mitchell's theory further postulates that $\Delta \mu H$ or one of its components is the immediate driving force for the inward movement of 'actively' transported substrates; particularly the transport of positively charged compounds such as K^+ are thought to be coupled with $\Delta \psi$.

The general terms of Mitchell's hypothesis [5—7] as as experimental evidence derived from *Escherichia coli* [11—13] indicate that the elements of the respiratory chain contribute significantly to $\Delta \mu H$ generation in aerobic conditions. Such a contribution appears to be negligible in the particular case of PG3. This conclusion stems from the fact that DCCD blocks $\Delta \psi$ generation and the resulting K^+ accumulation in aerobic conditions. Such inhibition does not take place in other bacterial systems where $\Delta \mu H$ generation results from respiratory activity [9,10].

Uncoupling agents clearly failed to modify K^+ distribution and $\Delta \psi$ unless valinomycin was present. This was unexpected. Mitchell's hypothesis states that agents which specifically increase H^+ permeability should, per se, short-circuit $\Delta \mu H$, and particularly $\Delta \psi$. Such a failure is all the more surprising as Fig. 3 shows that FCCP conducts protons across the mycoplasma cell membrane. This observation bears a striking resemblance to that expressed by Karlish et al. [33] who observed that, in chloroplasts, $\Delta \mu H$ is insensitive to high concentrations of FCCP, but is dissipated by a mixture of both FCCP and valinomycin. Further experiments are needed to establish whether this observation constitutes a clear inconsistency with regards to Mitchell's hypothesis or if it is only a matter of uncoupling efficiency in these particular mycoplasma membranes, containing a high proportion of lipids negatively charged. In any case, the simultaneous dissipation of $\Delta \psi$ and chemical K^+ gradient produced by a mixture of FCCP and valinomycin, again highlights the close connection between both processes.

In conclusion, although some aspects of the results including the limited extent of ATPase sensitivity to DCCD and the mode of action of uncoupling agents, call for further clarification, the present data indicate that the chemiosmotic phenomenon plays a crucial role in mycoplasma, judging by the properties of the K^+ transport system.

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