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Conditions modulating the ionic selectivity of transport by monensin examined on *Enterococcus hirae (Streptococcus faecalis)* by ^{23}Na-NMR and K^+ atomic absorption

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Factors likely to modulate the ionic selectivity of monensin were examined on *Enterococcus hirae* (*Streptococcus faecalis*) in two states previously characterized: the resting (de-energized) cell and the active (energized) cell. Internal and external Na^+ were followed by corresponding ^{23}Na-NMR resonances, K^+ concentrations were measured by atomic absorption. For a given cellular population of de-energized cells, the apparent transport rates and the final cationic concentrations reached at the steady state were decreasing with the ionophore dose. Monensin was selective for sodium only at low concentrations, in the range 1 mM–10^{-4} mM the transport was depending on the effective cationic gradients. Comparison of the activity curves for two cell populations ($7 \cdot 10^9$ and $7 \cdot 10^{10}$ cells/ml) showed the importance of the ratios of monensin/mg phospholipid and also of the ratios of external/internal volumes. On energized cells, except for low monensin concentrations, the main effect was a K^+-induced efflux and not a Na^+ influx. Two factors were modulating the resulting selectivity of this ionophore: the response of the intrinsic bacterial carriers and the generation of the gradients (mainly the external pH) which were favourable to a K^+/Na^+ transport. Once again the results obtained for two cell populations could be compared, the determining factors were the ratio external/internal volume and the generation of the pH gradient.

I. Introduction

Monensin, a carboxylic polyether antibiotic is well known for transporting cations across biological membranes as a mobile carrier, by a proposed antiport H^+/cation mechanism [1,2] (Fig. 1). Biochemists widely use it as a selective tool to modify Na^+ gradients. Despite its wide application in biology, the preferential transport by this ionophore of sodium over potassium has mainly been studied on model systems and rarely in living cells.

On models, its selectivity was established by measuring relevant constants for the different steps involved in the transport process. The pioneering work of Pressman [3] on a two-phase system (water-toluene/butanol, 70:30), also used by Gaboyard et al. [4] and recently improved by Hebrant et al. [5], clearly showed that the equilibrium constant for the heterogenous exchange reaction was in favour of Na^+/K^+. The development of NMR methods such as saturation transfer [6,7] and line broadening measurements [8,9,10] applied to large unilamellar vesicles (LUV) in the presence of aqueous shift reagents, afforded measurements of the apparent

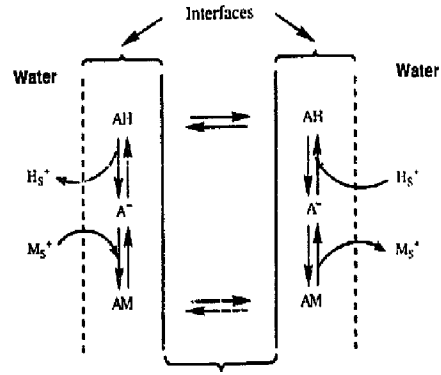

Fig. 1. Transport mechanism postulated in a model membrane for an antiport H^+/M^+ with monensin AH. S stands for 'solvated species'.

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stability constants for the ionophore-metal complex, and of the rate constants for the formation and dissociation of the complex at the membrane/water interface. Though K^+ on its own was transported more rapidly, Na^+ formed a more stable complex; thus monensin selectively transported Na^+/K^+ [10]. This selectivity was also shown in planar black lipid membranes (BLM) by a specific method and found to be 16 ± 4 for Na^+ versus K^+ [11,12]. All these studies were carried out without Na^+ and K^+ being directly in competition during the measurements. Bolte et al. [13] undertook to measure ionic fluxes as a function of pH in a three-phase system (water/chloroform/water) with these cations simultaneously present in one aqueous compartment. At pH 7, the observed flux was very weak for K^+ while maximum for Na^+. Thus on artificial membranes all the results support monensin selectivity in favour of Na^+ over K^+. But is this indication of ion-binding preference and transport still operative 'in vivo'?

For living cells the situation is far more complex, because of: (i) possible interference of the intrinsic transport systems of the cell, (ii) Na^+ and K^+ being in competition in both the aqueous compartments separated by the membrane and asymmetrically distributed in concentration, in the usual way:

$$[Na]_{in} \ll [K^+]_{in}, [Na^+]_{out} \gg [Na^+]_{in},$$

$$[K^+]_{out} \ll [Na^+]_{out}, [K^+]_{out} \ll [K^+]_{in},$$

$$pH_{in} > pH_{out}.$$

To our knowledge these complex experimental conditions have never been reproduced in model systems like vesicles and bilayer membranes. In order to approach this situation more closely we undertook a study on de-energized and energized *Enterococcus hirae (Streptococcus faecalis)* cells [14,15]. This enabled us to analyse the ionophore effect and bacterial response in the presence of real ionic gradients.

On de-energized cells we showed that with a high concentration of monensin, the induced cation movements were linked to the existing ionic gradients for K^+ and Na^+. On energized cells the greatest damage caused to the bacteria was a large K^+ efflux. Our results were consistent with studies performed on other bacteria [16,20] and erythrocytes [21,22], namely that the high selectivity of the ionophore for sodium as measured in model physico-chemical systems was not a determining factor in vivo.

The aim of the present work was to study factors likely to modulate the ionic selectivity of monensin, so as to determine 'directions for use' for a real sodium-selective tool for biochemistry. In particular, we investigated the influence of the dose of monensin on the resulting apparent selectivity. The definition of dose for a membrane effector needs to be clarified: it may be considered as either the effector concentration in the bulk phase (M/ml) or in the membrane (M/mg of phospholipid or M/number of cells). Both were studied on de-energized and energized cells. Na^+ and K^+ movements were followed by ^{23}Na-NMR and atomic absorption, respectively. For potassium, ^{39}K-NMR had previously been shown to be not sensitive enough for our system [14].

II. Material and Methods

Ionophore. Pure monensin A was prepared in our laboratory [14]. It was used in its acid form in ethanol solution.

Preparation of Enterococcus hirae (Streptococcus faecalis) CIP 58.55. De-energized cells were prepared as previously described [14], except for the resuspending medium, in which the phosphoric acid concentration was lowered from 200 μl/l to 100μl/l. After de-energization in choline buffer, NaCl was added to the bacterial suspension to 25 mM. We used a previously described method [23] to measure the protein concentration (20 mg/ml of bacterial suspension, $7 \cdot 10^{10}$ cells/ml; or 2 mg/ml, $7 \cdot 10^9$ cells/ml).

NMR spectroscopy. Spectra were recorded using a Bruker MSL 300 spectrometer at 21°C, with a 90° pulse (2K data points). 2 ml of cell suspension was transferred to 10-mm diameter tubes and 200 μl of D_2O was added for shimming.

^{23}Na-NMR spectra were accumulated at 79.39 MHz in 2-min blocks (7.5 ms pulse, 0.2 s repetition time, 300 scans). No line broadening was applied, allowing direct measurements of the sodium area (see ref. 14). The shift reagent was Dy^{3+} $(choline)_3^{3+}$ $(TTHA)^{3-}$ Cl_3^{3-} prepared according to Ref. 24 and used at a final concentration of 10 mM in the cell suspension.

Atomic absorption. K^+ content of the bacteria was determined by atomic absorption as follows. 20 μl NaCl 1 M was added to 400 μl of bacteria resuspended in choline buffer (20 mg protein/ml or 2 mg protein/ml). Bacteria were incubated at 21°C in the presence and absence of monensin. After incubation, bacteria were separated by rapid centrifuging. The pellet was washed twice with a few drops of water which were quickly aspirated. It was then digested with formic acid for 48 h at 37°C and analysed.

The external pH was measured with a glass pH electrode.

III. Results and Discussion

A. De-energized cells

Under our conditions the internal ion content of bacteria was 3 mM Na^+, 50–70 mM K^+ (pH 6.9) and

the external medium was 25 mM NaCl, 5 mM KCl (pH 7.3).

1. Modulation of transport selectivity according to amount of added monensin

The decrease in K^+ internal concentration is shown in Fig. 2. We shall focus on the experiments performed with $7 \cdot 10^{10}$ cells/ml. The observed response was sigmoidal: below 10^{-5} mM, monensin was inactive, from 10^{-5} mM to 10^{-2} mM the change in K^+ concentration increased with the monensin dose, and finally above 10^{-2} mM a plateau was reached. This type of curve can be compared to those obtained by Chow et al. [20] who measured lactate production and glucose consumption for increasing monensin concentrations in *S. bovis*. To some extent, the plateau observed may arise from zero order of exchange rate constants as it was measured on LUV for high concentrations of monensin. This might be explained by 'saturation' of the membrane with monensin; beyond a threshold monensin/lipid ratio, the ionophore can no longer be incorporated in the membrane. From this curve of activity the monensin concentrations 1 mM, 10^{-2} mM, 10^{-3} mM and 10^{-4} mM were selected and incubated for 45 min with de-energized cells ($7 \cdot 10^{10}$ cells/ml) in the absence of glucose. Representative kinetics of Na^+ influx and K^+ efflux are presented in Figs. 3A and 3B, respectively. The 'apparent transport rate' of Na^+ and K^+ increased with monensin concentration; for 1 mM, the Na^+ influx or K^+ efflux was instantaneous though the steady state was reached after 45 min for lower concentrations; this modulation was more marked for K^+ than for Na^+. It is of interest that for dynamic

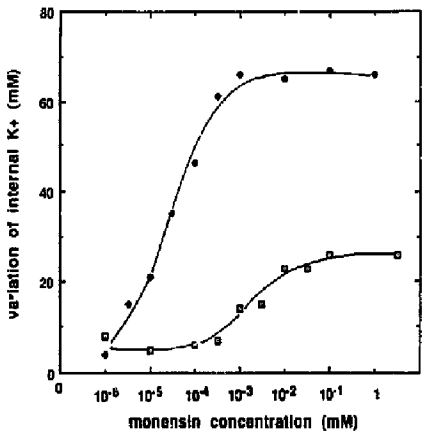

Fig. 2. Curve of activity of monensin. After incubation of monensin to de-energized cells for 45 min, the variation of intracellular K^+ was measured by atomic absorption. Two bacterial concentrations were tested: ⊡, $7 \cdot 10^{10}$ cells/ml and ●, $7 \cdot 10^9$ cells/ml.

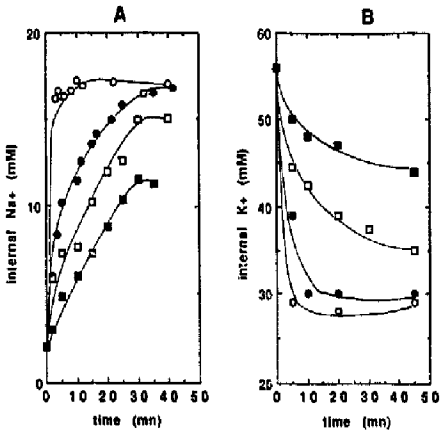

Fig. 3. De-energized cells ($7 \cdot 10^{10}$ cells/ml) were incubated with increasing concentrations of monensin (○, 1 mM; ●, 10^{-2} mM; □, 10^{-3} mM; ■, 10^{-4} mM), intracellular Na^+ (A) and K^+ (B) concentrations were measured by ^{23}Na-NMR and atomic absorption, respectively.

studies performed on model membranes [5,10,11], the rate constants for Na^+ transport also increased with the dose of monensin.

The net Na^+ or K^+ transport stopped when an ionic equilibrium (steady state) was reached. Actually the change of Na^+ or K^+ concentration decreased slightly with the dose of monensin (Table I). From these results, it is clear that Na^+ selectivity was only effective for the lowest concentration of monensin (10^{-4} mM); in the other cases Na^+ and K^+ movements were driven by the ionic gradients present in the bacterial preparation.

This predominant K^+ efflux cannot be due to the intrinsic K^+ carriers of *E. hirae* in response to modifications of Na^+ and H^+ gradients previously induced by monensin. Indeed, in opposition to secondary

TABLE I

Variation of the internal Na^+ and K^+ concentrations mediated by monensin on de-energized cells

De-energized *E. hirae* cells ($7 \cdot 10^{10}$ cells/ml) were incubated with variable doses of monensin during 45 min in choline buffer (100 mM choline chloride, 2 mM EDTA, 5 mM $MgSO_4$, 40 mM Mes, 100 μl/l H_3PO_4, 25 mM NaCl, adjusted to pH 7.3 with choline hydroxide) in absence of glucose.

Dose of monensin	ΔNa^+ [a]	ΔK^+ [a]
1 mM	14 mM	−23 mM
10^{-2} mM	14 mM	−23 mM
10^{-3} mM	13 mM	−19 mM
10^{-4} mM	10 mM	−3 mM

[a] These values were determined from Figs. 3A and 3B. $[Na^+]_{45min} - [Na^+]_{0min} = \Delta Na^+$; $[K^+]_{45min} - [K^+]_{0min} = \Delta K^+$.

H^+/K^+ antiporters described in many bacteria, *E. hirae* exhibits a distinctive pattern to extrude potassium via an H^+/K^+ ATP driven system [25] or a Na^+/K^+-ATPase [26]. Consequently de-energized cells were unable to expell K^+ per se, K^+ was effectively transported by monensin preferentially to Na^+.

pH changes were determined simultaneously by ^{31}P-NMR (data not shown), internal pH was fairly stable around pH 6,9 ± 0.1 resulting from good compensation of Na^+ and K^+ fluxes and from good buffering of the internal medium.

2. Influence on monensin selectivity of the ratio monensin / number of cells

The changes of K^+ internal concentration in the presence of increasing concentrations of monensin were compared for two bacterial populations (Fig. 2). The general sigmoidal shape of the curves of activity were comparable; thus the previous remarks for $7 \cdot 10^{10}$ cells/ml hold for $7 \cdot 10^9$ cells/ml. However, these curves were noticeably shifted on both axes: (i) the thresholds of the active dose and of the plateau were shifted from 10^{-5} mM ($7 \cdot 10^{10}$ cells/ml) to 10^{-6} mM ($7 \cdot 10^9$ cells/ml) and from 10^{-2} mM ($7 \cdot 10^{10}$ cells/ml) to 10^{-1} mM ($7 \cdot 10^9$ cells/ml), respectively; (ii) the amplitude of $[K^+]$ change was much higher when the cell concentration was lower for instance, with 1 mM monensin, $\Delta K^+ = 68$ mM for $7 \cdot 10^9$ cells/ml instead of $\Delta K^+ = 28$ mM for $7 \cdot 10^{10}$ cells/ml. It can be noticed that for $7 \cdot 10^{10}$ cells/ml, not all the K^+ was released by addition of high concentrations of ionophore.

These results are surprising as a simple shift of the doses proportional to the ionophore/membrane ratio would have been expected. In fact a change of cell concentration induces a change in the monensin/phospholipid ratio and also a modification of the external/internal volume ratio R ($R = 368\ \mu l/1832\ \mu l$ for $7 \cdot 10^{10}$ cells/ml, $R = 37\mu l/2163\ \mu l$ for $7 \cdot 10^9$ cells/ml). As a consequence, with 10^9 cells/ml (lower concentration), more K^+ must be expelled out of the bacteria to reach the same ionic equilibrium (steady state) than with 10^{10} cells/ml.

To conclude: in addition to the modulation of ionic selectivity by monensin concentration, the influence of monensin/number of cells ratio is crucial.

B. Energized cells

Bacteria were incubated with monensin for 45 min, and 40 mM glucose was then added for 30 min. Under these conditions, cells were reactivated and the response of the intrinsic carriers could be tested.

1. Influence of monensin concentration

Na^+ and K^+ kinetics in the presence of glucose are presented in Figs. 4A and 4B, respectively.

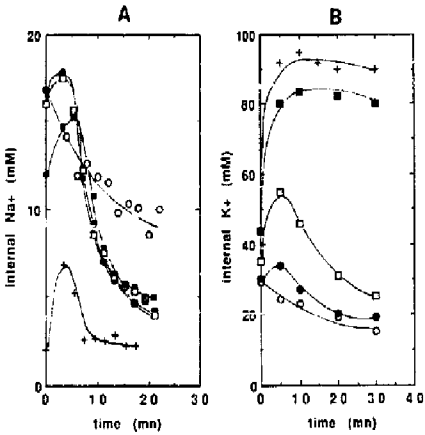

Fig. 4. After incubation with monensin (○, 1 mM; ●, 10^{-2} mM; □, 10^{-3} mM; ■, 10^{-4} mM) or without monensin (+) for 45 min, glucose was added to the bacterial suspension ($7 \cdot 10^{10}$ cells/ml). Na^+ (A) and K^+ (B) kinetics were followed by ^{23}Na-NMR and atomic absorption, respectively.

For Na^+ kinetics, two main situations were observed: with 1 mM of monensin, as previously shown [15], the normal Na^+ gradient was not recovered. For lower concentrations, the observed Na^+ kinetics were much more similar to the reference. Sodium was first pumped inside the bacteria and then expelled outside the cell. We had shown previously that this entry was dependent on the phosphate concentration [27].

K^+ kinetics were rather different; a more gradual change with dose was observed: the only 'normal' K^+ gradient was reached with 10^{-4} mM; for 10^{-2} and 10^{-3} mM bacteria started pumping back K^+ without success, possibly because they ran out of ATP after a few minutes although glucose was till available (initial ATP concentration: 2.3 mM, was consumed to fight the monensin effect); for 1 mM the K^+ leakage was accentuated.

In spite of the claimed Na^+ selectivity of monensin except for 10^{-4} mM, bacteria seem to regulate Na^+ gradients better than K^+ ones.

In addition to this possible intrinsic 'active' modulation by bacterial carriers, it can be argued that during glycolysis, experimental conditions are more and more unfavorable to a Na^+ selectivity: after addition of glucose a large pH gradient ($pH_{ext} \ll pH_{int}$) was developed [15]. As a K^+ gradient still existed (whereas Na^+ gradient was lower) the experimental conditions thus established facilitated a H^+/K^+ exchange (these pH conditions did not exist for the de-energized cells). pH influence must be taken into account as already suggested by Chow and Russel [20].

2. Comparison of Na^+ and K^+ movements for two bacterial populations

As stated above, the ratio monensin/number of cells can change the resulting cation selectivity. In this work only one dose of monensin was tested for two bacterial concentrations, but the results were striking. In the presence of glucose, opposite results were obtained for K^+ changes (Fig. 5A): with 10^9 cells/ml, K^+ was partially pumped back to the cell in spite of the initial larger ΔK^+, while with 10^{10} cells/ml, K^+ leakage was accentuated.

This unexpected result can be explained if once again the crucial problem of the pH variation is considered: the external pH was measured during glycolysis (Fig. 5B) with and without monensin, in the presence of 10^{10} or 10^9 cells/ml. With diluted cells, the external pH change was very low. On the contrary, with concentrated cells a large pH gradient was developed. The lower pH gradient obtained with 10^9 cells/ml can result from both less glucose degraded (less lactate produced) and from a more efficient buffering effect of Mes. As explained earlier, a large pH gradient can induce an outward K^+ movement, the absence of this gradient is unfavorable for K^+ transport by monensin, in this situation it may be that *E. hirae* carriers can partially restore a normal K^+ gradient.

This experiment indicates that the generation of ionic gradients induced by the metabolism of the bacteria per se varies with cell concentration. The regulation of these gradients is also modulated by the ratio external volume/internal volume and consequently can interfere differently with ionophore transport.

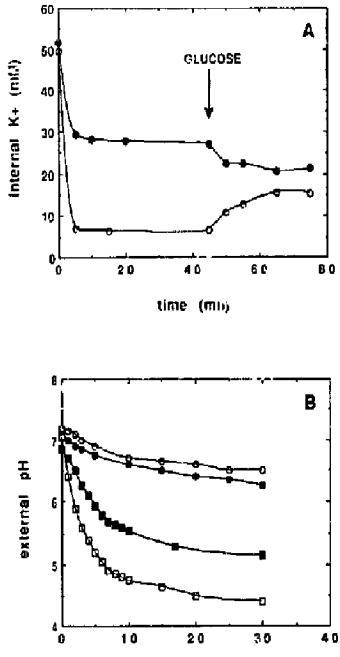

Fig. 5. (A) 1 mM monensin was added to de-energized cells (●, $7 \cdot 10^{10}$ cells/ml and ○, $7 \cdot 10^9$ cells/ml), after 45 min of incubation, 40 mM glucose was added to the bacterial suspensions. The variations of K^+ concentrations were measured by atomic absorption. (B) After incubation with 1 mM monensin for 45 min (■, $7 \cdot 10^{10}$ cells/ml and ○, $7 \cdot 10^9$ cells/ml) or without monensin (□, $7 \cdot 10^{10}$ cells/ml and ●, $7 \cdot 10^9$ cells/ml, glucose was added (time zero), the variation of the external pH was recorded with a glass pH electrode.

IV. Conclusion

In this work we have shown that the claimed Na^+ selectivity of monensin is not always effective with living cells. Both on de-energized cells and energized cells, the amplitude of the initial ionic gradients (Na^+, K^+, H^+) and the concentration of the monensin modulated the resulting selectivity. The experiments performed on two bacterial populations showed that the external/internal volume ratio was a determining factor. In the presence of glucose the situation was even more complex because of the interference of ionophore/bacterial carriers. Another crucial problem was the evolution of the ionic gradients (especially pH gradient) after addition of glucose that could modify the activity of the ionophore per se.

These results suggest that monensin activity should be checked (in particular by measuring Na^+ and K^+ concentrations) for all new experimental conditions.

Monensin is a selective tool for Na^+ transport under only very restricted conditions. Experimentation must be very carefully monitored when studies based on different cell concentrations (because of sensitivity) are performed in parallel or when results are to be compared with the literature.

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