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PHOSPHATE-DEPENDENT SODIUM TRANSPORT IN *S. FAECALIS* INVESTIGATED BY ^{23}Na AND ^{31}P NMR

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Na^+ movements in *S. faecalis* were studied by ^{23}Na NMR. They proved to be dependent on phosphate concentration in the buffer during the de-energization step. K^+ and H^+ were also studied respectively by potentiometry and ^{31}P NMR and were shown not to be implicated. For de-energized cells the internal phosphate concentration, on the contrary, was directly linked to the external phosphate contained in the buffer. The experiments showed a Na^+/P_i dependence in this prokaryote so far known only in eukaryotes.

Bacteria can extrude sodium ions by various active mechanisms. Kakinuma and Harold showed that *Streptococcus faecalis* had two independent Na^+ carriers : an inducible Na^+/K^+ ATPase and a constitutive Na^+/H^+ antiporter (1-3). The recent development of shift reagents allows discrimination of the ^{23}Na NMR resonances for the intra and extra-cellular sodium, thereby facilitating the study of its transport in living cells (4-11). This enabled us to investigate the modifications of ionic gradients induced on *S. faecalis* by monensin (12,13), a well known bacterial ionophore claimed to selectively transport Na^+. In the course of this work we incidentally observed by ^{23}Na NMR that for cells not treated by monensin, sodium movements were dependent on phosphate concentration in the following conditions : previously de-energized cells, reactivated by glucose additions in different buffers, showed responses of the intrinsic Na^+ carriers which were modulated by the phosphate concentration in the buffer.

In order to understand this phenomenon, we determined the intracellular phosphate concentration by ^{31}P NMR, a non invasive method allowing the phosphate transport to be followed, in addition to the usual pH measurements (4,14-16).

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The study was completed by simultaneous determination of the internal pH (^{31}P NMR) and K^+ movements (potentiometry). The Na^+/P_i relationship was clearly shown for the first time in this prokaryote cell.

MATERIALS AND METHODS

Preparation of *Streptococcus faecalis* CIP 5855 de-energized cells

Late logarithmic phase cells (A = 1.5) were washed twice by centrifugation at 8000 x g and resuspended in medium containing 100 µM choline chloride, 2 mM EDTA, 5 mM $MgSO_4$, 40 mM MES, adjusted to pH 7.3 with choline hydroxide, the phosphate and / or Na^+ concentrations were variable as stated in Table 1. Cells were left in this medium at 4°C overnight and washed twice again. Cells were kept at ice temperature before use. Protein concentration was determined classically (16).

NMR spectroscopy

Experiments were performed on a Bruker MSL 300 spectrometer at 21°C with a 90° pulse [2K data points]. 2 ml of cell suspension (20 mg protein/ml) were transferred to 10-mm diameter tubes and 200 µl of D_2O was added for shimming. ^{31}P NMR spectra were accumulated at 121.49 MHz in 2 min-blocks (8µs pulse, 0.3 s repetition time, 600 scans). Chemical shifts were referenced to external 85 % phosphoric acid. ^{23}Na NMR spectra were accumulated at 79.39 MHz in 2 min-blocks (7.5 µs pulse, 0.2 s repetition time, 300 scans). No line broadening was applied, allowing direct measurements of the sodium area. The shift reagent was $(choline^{3+})Dy^{3+}$ $(TTHA^{3-})$ (3 choline chloride) prepared according to ref. 14 and used at a final concentration of 10 mM in the cell suspension. The $choline^+$ counterion avoided any sodium being brought by the shift reagent. This reagent was shown previously to be non toxic on *S. faecalis* (12). Internal Na^+ visibility being estimated to be 60% (12), a correcting factor was thus applied to the observed area. ^{23}Na spectra were collected every 2 minutes.

Atomic absorption

Internal K^+ content was measured as described previously (12).

K^+ potentiometry

The variations of the potassium content of the cells were determined by measuring the changes of the K^+ external medium with a selective electrode (Ingolg type 15 221 3000, for potassium) associated with a calomel reference electrode containing a secondary salt bridge filled with a solution of 100 mM NaCl. The cation electrode potential was calibrated with KCl solutions of known concentrations, prepared in the experimental buffer.

RESULTS

Streptococcus faecalis cells were de-energized in two types of buffer : The so-called "choline buffer" with no sodium content and the "choline sodium buffer" containing 25 mM NaCl. Two concentrations of phosphate were used for each buffer. After an overnight de-energization step, the internal Na^+, K^+ and H^+ concentrations were fairly constant in the absence of glucose, corresponding to the reached steady state, they were determined for each set of conditions and are reported in Table 1.

Table 1

Ionic parameters for de-energization of *S. faecalis* cells

De-energization buffer	External concentration				Internal concentration			
	[Na^+] mM	[K^+] mM	pH	[Pi] mM	[Na^+] mM	[K^+] mM	pH	[Pi] ≠
"choline buffer"								
Choline-5	none* [+25]	none	7.0	5	3.5	93	6.7	(68)
Choline-20	none* [+25]	none	7.1	20	3.2	114	6.6	(1015
"choline sodium buffer"								
Choline-Na^+-10	25	none	7.4	10	27.9	71.6	7.0	(180)
Choline-Na^+-20	25	none	7.2	20	25.9	75.0	7.0	(348)

*When "choline buffer" was used, NaCl was added to the bacterial suspension after de-energization up to 25 mM.
≠ Internal Pi concentrations are expressed in arbitrary units which corresponds to ^{31}P NMR area [Pi + teichoic acids].
The above-mentioned ionic parameters were measured after an overnight de-energization step, for Na^+ these values correspond to t = 0 in figure 1.
Uncertainties: ± 0.1 unit for pH measured by NMR ; ± 15% for [Na^+] and [Pi] measured by ^{23}Na and ^{31}P NMR respectively; ± 15% for [K^+] measured by atomic absorption.

Although the internal concentrations of K^+ (90 mM) and H^+ (pH = 6.6) were fairly constant whatever the experimental conditions, the presence of 25 mM NaCl in the de-energization buffer led to sodium loaded cells ($[Na^+]_{in}$ = 26 mM). This de-energization process is usual for studying active transport mechanisms. Addition of glucose to the de-energized cells reactivated the intrinsic cation carriers of the bacteria. During the glycolysis, Na^+ movements were followed by ^{23}Na NMR, the resulting kinetics are reported in figure 1. The main feature which emerges is the dependance of the sodium influx upon the phosphate concentration used during the de-energization step ; this observation was highly reproducible and only observed in the presence of glucose, suggesting an active process. When the phosphate concentration was lower, the cells pumped Na^+ during the first minutes and then this it was expelled down to a rather low internal concentration. Interestingly, this also occured with the "choline sodium buffer" for which the cells were loaded with Na^+.

From these results it was clear that cells after de-energization behave differently depending on the phosphate concentration in the buffer. We looked for significant features as shown in table 1.

As P_i was involved, we measured its intracellular concentration from ^{31}P NMR spectra (figure 2). NMR conditions did not allow quantitative determinations because

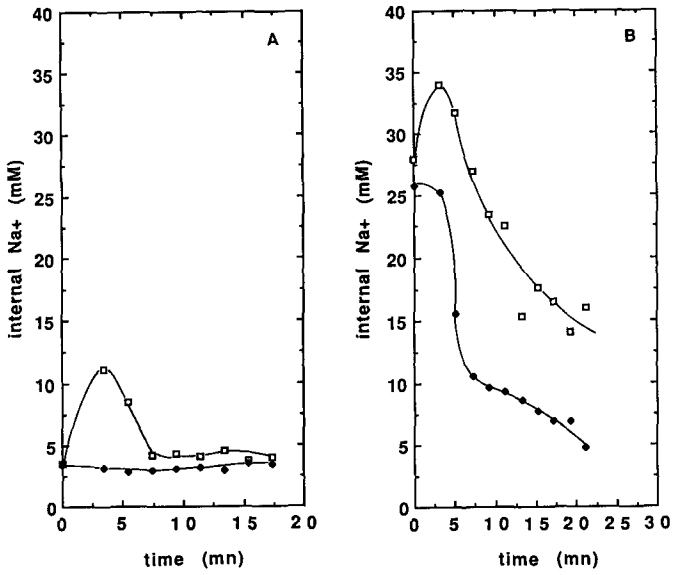

Figure 1 . After an overnight de-energization at 4°C, the resting cells were incubated with 40 mM glucose (t = 0). Na^+ concentrations were measured by Na^+ NMR (79.39 MHz) with (choline^{3+}) Dy^{3+} ($TTHA^{3-}$) (3 choline chloride) as shift reagent. ^{23}Na NMR spectra were collected in 2 min blocks (300 scans).
A: *S. faecalis* cells were de-energized in "choline buffer" with 5mM [Pi] (□)or 20 mM [P_i] (◆) **B**: *S. faecalis* cells were de-energized in "choline sodium buffer" with 10 mM [P_i] (□)or 20 mM [P_i] (◆).
In the absence of glucose, the internal Na^+ concentrations remained constant to their initial values (t = 0) and may be considered as controls.

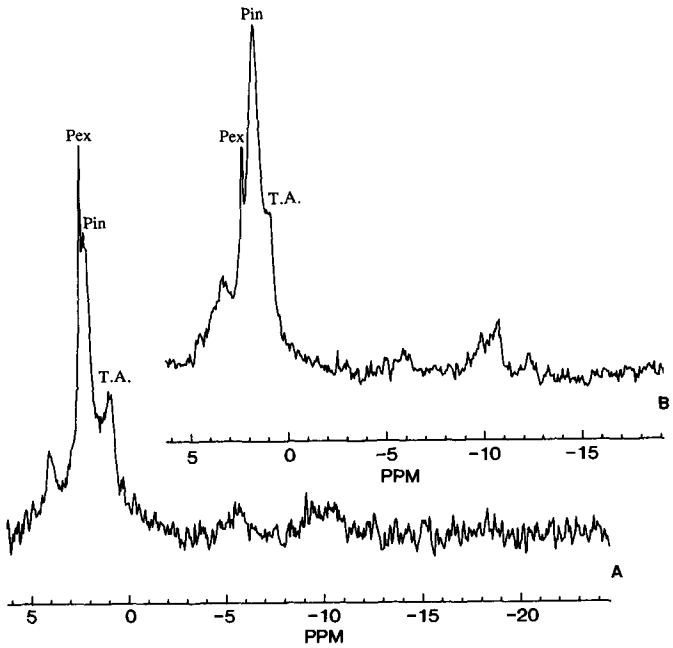

Figure 2 . ^{31}P NMR spectra (121.5 MHz) of *S. faecalis* cells (20 mg protein/ml) de-energized in " choline buffer" containing 5 mM [P_i] (**A**) or 20 mM [P_i] (**B**).
T.A. teichoic acids. Internal Pi concentrations are evaluated from ^{31}P NMR area [P_{in}+T.A.].

teichoic acids (δ = 0.84 ppm), components of Gram (+) cell wall (12), contributed to the internal phosphate signal area, but this may be taken as a constant factor since the bacterial concentration was constant. The values reported in table 1 for the internal phosphate are thus expressed in arbitrary units, but they are comparative. Obviously the internal phosphate concentration increased when the external phosphate was increased in the de-energization buffer. As a result, the internal phosphate proved to be an important variable factor for de-energized cells which could be correlated indirectly to sodium transport.

Activity of the Na^+, K^+, H^+ intrinsic carriers (1-3, 19-21) could also contribute to the observed Na^+ movements. Consequently we also determined the K^+ and H^+ compartmental variations. The efflux of K^+ was followed by potentiometry (K^+-selective electrode). No difference could be detected while phosphate concentration was increased in the buffer. Similarly, the time-course of the internal pH measured by ^{31}P NMR shown no significant variation. Clearly, the dependence on phosphate concentration was only observed for the sodium movement.

DISCUSSION AND CONCLUSION

The study of *S. faecalis* de-energized cells in buffers with increasing phosphate concentrations showed that :

i) Na^+, K^+ and H^+ intracellular concentrations were not affected, unlike internal phosphate which was increased ;

ii) In the presence of glucose, Na^+ transport was dependent on the phosphate concentration during the de-energization step. With low [P_i], Na^+ was first pumped in the cell, H^+ and K^+ being unchanged.

These experiments reveal indirect correlation between intracellular [P_i] and Na^+ transport. The following explanation is suggested : at the beginning of the glycolysis, phosphate is needed for the phosphorylating steps of the metabolism ; therefore if little phosphate is available in the cell (i.e. internal phosphate is low) bacteria have to pump phosphate from the external medium. The observed Na^+ intrance could thus be related to the phosphate movement. Harold et al. (22) have described the presence of a H^+-P_i symporter /or a OH^--P_i in *S. faecalis*. Na^+ movements could result from the activity of the Na^+/H^+ antiporter in response to the pH variation induced by phosphate transport. This would be consistent with the pH regulation observed whatever the phosphate concentration. Or, the presence of a Na^+/P_i cotransport might be envisaged. This type of carrier has been described for eukaryote cells (23-26), especially in yeast (27). As regards its Na^+ / K^+ ATPase (3) usually specific for eukaryotes*S. faecalis* is an exception. The presence of a Na^+ / P_i transport would add new elements to this first established peculiarity.

Further investigations in this stimulating direction are in progress, for this purpose ^{23}Na and ^{31}P NMR proved to be very helpful.

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