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Intracellular sodium content of a wall-less strain of *Neurospora crassa* and effects of insulin: a ^{23}Na-NMR study

N.J. Greenfield [1], C.N. Cherapak [2], F. Adebodun [2], F. Jordan [2] and J. Lenard [1]

[1] *Department of Physiology and Biophysics, UMDNJ-Robert Wood Johnson Medical School (at Rutgers), Piscataway, NJ and* [2] *Department of Chemistry, Rutgers University, Newark, NJ (U.S.A.)*

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^{23}Na-NMR has been used to investigate some factors influencing the sodium content of a wall-less strains of *Neurospora crassa*. The shift reagent Tm(DOTP)$H_2(NH_4)_3$ proved useful for this purpose, while several other reagents, previously used by others, were found to be unsuitable for use with these cells. When the cells were grown, washed and resuspended in medium containing sodium (25.3 mM), the intracellular sodium concentration was calculated to be 11.9 ± 1.4 mM. This value rose within two minutes of addition of glucose (100 mM), to > 14 mM. Preincubation of cells with insulin (100 nM) had a significant effect on the subsequent rate of sodium accumulation during the period 3–12 minutes following glucose addition. Insulin-treated cells showed a slow, continued accumulation of sodium during this period (+1.14 ± 0.39%/min), while control cells lost sodium very slowly (−0.63 ± 0.29%/min; *P* of difference = 0.005).

Introduction

The 'slime' variant of the filamentous fungus, *Neurospora crassa*, lacks a rigid cell wall and is therefore very useful as an experimental organism for the study of certain aspects of fungal metabolism. For example, it is easy to isolate plasma membranes [1] and vacuoles [2] from slime in high yields without the problems associated with removal of a cell wall. In addition, the isolation of subcellular particles and enzymes from the mutant is also a great deal easier than from the parent walled strain. Recently we have been characterizing the metabolism of slime, and its hormonal regulation using a variety of techniques [3–5]. As a part of these ongoing studies we desired to examine some of the factors which influence sodium homeostasis in this organism. Unfortunately, the classical technique which has been used to study sodium content in the wild-type parent organism [6] is not applicable to the study of slime because the wall-less cells are quite fragile and cannot be collected by rapid filtration on small pore membranes without breakage.

Correspondence: J. Lenard, Department of Physiology and Biophysics, Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, NJ 08854-5635, U.S.A.

Recently ^{23}Na-NMR has been successfully used to study the sodium content of intact cells in vivo. In order to use NMR to study sodium homeostasis it is necessary to distinguish intra- from extra-cellular sodium. The most widely used technique for doing this is to use a shift reagent. Shift reagents are non-penetrating compounds that contain paramagnetic metals bound to charged chelating agents; they therefore cause a chemical shift in the resonance arising from extracellular sodium [7,8]. In addition, the difference in the rates of relaxation of the resonances arising from sodium on the inside and outside of cells has been employed to resolve their NMR signals [9].

In this paper we compare the usefulness of several shift reagents for separating the resonances of intra- and extra-cellular sodium in the slime mutant of *N. crassa*. We also report the resting intracellular concentration of sodium in the slime mutant, and the effects of energizing the cells with glucose and oxygen.

N. crassa produces an insulin-like protein [18,19]. Moreover, the slime mutant of *N. crassa* possesses a membrane binding protein that is specific for mammalian insulin [20] and has been shown to respond to insulin in several ways, including: an increase in glycogen formation [4]; an increase in the yield of carbon dioxide from glucose [3]; and an increase in the rate of ethanol production [5]. We therefore decided to ex-

amine the effect of glucose and insulin on the sodium content of slime cells, because insulin has been shown to affect the sodium balance in a variety of mammalian tissues (for a review see Moore [21]).

Materials and Methods

Materials. Ethylenediaminetetraacetic acid (EDTA), ethylene glycolbis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA), triethylenetetramine-hexaacetic acid (TETHA), sodium tripolyphosphate (PPP), 2-(*N*-morpholino)ethanesulfonic acid (Mes), azolectin (soybean phosphatides), ATP, 5′-adenylyl methylenediphosphonate (AMPPCP) and 5′adenylylimidodiphosphonate (AMPPNP) and crystalline bovine insulin were obtained from Sigma Chemical Co. (St. Louis, MO). Potassium tripolyphosphate and dysprosium trichloride ($DyCl_3$) were obtained from Alfa Products (Danvers, MA). Methylene diphosphonate was obtained from Aldrich Chemical Co. (Milwaukee WI). Thulium (1,4,7,10-tetraazacyclodecane-*N*,*N*′,*N*″,*N*‴-tetramethylenephosphonate-$H_2(NH_4)_3$) [$Tm(DOTP)H_2(NH_4)_3$] was a generous gift from Dean Sherry from the University of Texas at Dallas.

Cell culture. Stock cultures of *Neurospora crassa*, strain FGSC 4761 (fz;sg;os-1(B135),A;V) were supplied by G. Scarborough (University of North Carolina, Chapel Hill NC), and were maintained routinely as described by McKenzie et al. [3]. For NMR experiments, cells (10^8) were inoculated into 50 ml of a supplemented defined medium consisting of Vogel's minimal medium [10] containing 330 mM sorbitol, supplemented with trace elements, vitamins, amino acids and 2% sucrose (SDM) [3] and grown for 18 h. After growth the cells were allowed to sit in their conditioned medium without aeration at 23° C until the NMR experiments could be performed (1 to 6 h). The cells were resuspended and washed in Vogel's minimal medium [10] minus trace elements and biotin, containing 330 mM sorbitol (MM) directly before the ^{23}Na-NMR spectra were obtained. For some measurements cells were resuspended and washed in MM in which sodium citrate was replaced with potassium citrate, or in 100 mM Mes-Tris buffer, containing 330 mM sorbitol (pH 5.8). They were then resuspended in medium containing 10% D_2O and the required shift reagent for the NMR measurements.

For measurements of the effect of insulin and glucose, cells were preincubated for 15 min in MM [10] in the presence or absence of 100 nM insulin, at 23° C in the NMR spectrometer. Cells were oxygenated with 95% O_2/5% CO_2, delivered to the bottom of a sample tube via tygon tubing at a rate of one bubble (2-mm diameter) per second, which was sufficient to prevent settling of the cells. A basal spectrum was then collected. Glucose (100 mM) was subsequently added, and spectra were collected for five periods of 2.67 min each (1600 free induction decays).

Preparation of shift reagents. The shift reagents containing dysprosium were all prepared in a similar fashion. 1 mmol of $DyCl_3$ was dissolved in approx. 3 ml of water and mixed with a 2-fold excess of chelating agent in 3 ml of water. The compounds were sonicated together in a bath sonifier until the mixtures dissolved. If necessary the pH values were adjusted to approx. 7 with either concentrated Tris base or HCl, and the final volume was adjusted to 10 ml to give a 0.1 M stock solution of shift reagent, and clarified by filtration through a 0.45 μm filter. The stock solutions were added to the cell suspensions to give the desired concentration of shift reagent, usually between 2 and 10 mM.

$Tm(DOTP)H_2(NH_4)_3$ was dissolved in MM [10] plus sorbitol at a concentration of 5 mM, and washed cells were directly resuspended in this medium, giving a final concentration of the reagent in the medium of 2.5 mM.

Preparation of vesicles. Large unilamellar vesicles of azolectin in 0.1 M Na_2SO_4 were prepared as described by Pike et al. [8].

NMR measurements. Initial ^{23}Na-NMR measurements were obtained at 52.9 MHz using a Bruker WP-200 SY spectrometer operating in the pulse Fourier transform mode at 23° C in 10-mm NMR tubes. The spectral width was 50000 Hz, the pulse width was 15 μs with a 0.041 s acquisition time. To improve the signal to noise ratio in the spectra of the intact cells exponential multiplications of the free-induction decay were performed with line broadenings of 3 Hz. When $Dy(EGTA)_2$ and Dy(AMPPNP) were employed as the shift reagents the resolution of the internal and external sodium signals were enhanced by employing Gaussian multiplication. Subsequent ^{23}Na-NMR measurements were obtained at 105.8 MHz on a Varian XL-400 MHz instrument in 5-mm NMR tubes. For these measurements the spectral width was 10000 Hz and the pulse width was 12 μs with a 0.1 s acquisition time. In these measurements the line broadening was 10 Hz. For all NMR measurements the fields were locked on the deuterium resonance of D_2O and chemical shifts were referenced to 0.1 M NaCl. During the collection of NMR spectra cells were oxygenated with 95% O_2/CO_2 as previously described [5]. T_2 measurements were performed as described by Okerlund and Gillies [9].

Calculation of Intracellular sodium concentration. The intracellular content of sodium was estimated from the area of the signal arising from the intracellular compartment, relative to the total sodium in the NMR tube which was measured relative to an external standard of sodium chloride. The intracellular volume of the slime cells was estimated assuming a volume of 3 μl/mg as determined by Schulte and Scarborough [11] using the method of Lowry et al. [12] to determine the protein

concentration. The protein concentration used for the NMR measurements ranged from 20 to 60 mg/ml.

Results

Relaxation rate of intracellular sodium

In initial studies it was attempted to resolve the intracellular and extracellular sodium resonances by examining their T_2 relaxation rates in the absence of an added shift reagent as described by Okerlund and Gillies [9]. In the absence of slime cells, the sodium signal in MM was 0.031 s. In the presence of cells the T_2 decreased, but only one relaxation time was detected and the relaxation time decreased with increasing cell protein concentration. The T_2 was 0.010 sec when the cell protein concentration was 25 mg/ml and dropped to 0.0055 s at a cell protein concentration of 50 mg/ml. The change in the T_2 value as a function of cell concentration indicates that the cells affect the relaxation time of sodium in the extracellular medium. Since only one relaxation rate was observed it appears that the difference in relaxation rate between the interior and exterior of the cells is too small to distinguish the intra- and extra-cellular resonances and/or that the intracellular sodium signal is too small a percentage of the extracellular signal to detect by T_2 measurements.

Shift reagents

Since relaxation rates proved unsuitable for distinguishing intra and extracellular sodium in slime cells, it was necessary to find an appropriate shift reagent. Three major classes of water stable shift reagents have been described in the literature. The first class are complexes of paramagnetic ions with highly charged cations such as tripolyphosphates [7]. The second involves salts of paramagnetic ions complexed with chelating agents of the EDTA family [13,14]. More recently Sherry et al. [15] have described a third class of shift reagents consisting of paramagnetic salts (Dy or Tm) complexed with DOTP.

Representatives of each of these classes of reagents were tested to see whether they would be useful for resolving intracellular from extracellular sodium in slime cells.

Most of the reagents reported in the literature for use with other cell systems proved unsuitable for slime. Dysprosium tripolyphosphate was rapidly hydrolysed by extracellular phosphatase, and thus could not be used in the presence of the *N. crassa* cells. Non-hydrolyzable analogs of $Dy(PPP)_2$ prepared with either methylene diphosphonate or AMPPCP broadened the sodium resonance but caused only small changes in the chemical shift and therefore were not useful as shift reagents. $Dy(ATP)_2$ proved more resistant to phosphatase than $Dy(PPP)_2$ and the compound was equal in resolving power to the tripolyphosphate in vitro. However, results obtained with this reagent in experiments involving intact cells were not reproducible. On some occasions when $Dy(ATP)_2$ was added to the cells a small intracellular resonance appearing at the position of uncomplexed sodium was clearly resolved from a large external shifted resonance. This result showed that the rate of exchange of sodium between the internal and external compartments was slow on the NMR time scale. However, the extracellular resonance occasionally showed severe broadening suggesting that $Dy(ATP)_2$ was interacting with the cells, possibly causing local field inhomogeneities. In addition, on some occasions when cells were examined in the presence of $Dy(ATP)_2$ only one signal was observed, suggesting that sodium was in fast exchange between the inside and outside of the cell. The exact factors influencing the rate of exchange of sodium in the presence of the ATP containing shift reagent could not be determined. $Dy(AMPPNP)_2$ neutralized with Tris buffer, was also equal in resolving power to $Dy(PPP)_2$ and was not hydrolysed by the *N. crassa* cells. Signals from internal and external sodium were clearly resolved using this shift reagent and sodium appeared to be in slow exchange between the inside and outside of the cells when this reagent was employed. The reagent did not appear to cause cell lysis and the NMR signals were constant for 15 min in the presence of the reagent and the cells remained viable over the course of the NMR experiments. The reagent was useful in determining the intracellular sodium content in freshly prepared, washed cells at rest (see below). However, since AMPPNP is a metabolic inhibitor, further studies using this shift reagent could not be carried out.

Shift reagents prepared with dysprosium and chelating agents such as EDTA, EGTA, TETHA, and NTA gave only small shifts, and in the presence of cells usually did not resolve the intracellular signals. Moreover, the cells rapidly lost sodium in the presence in some of these reagents. For example, the addition of $Dy(EDTA)_2K_8$ to cells which had been washed in sodium-free medium caused a rapid efflux of the intracellular sodium. The addition of $Dy(EGTA)_2^{8-}$ which was neutralized with Tris buffer also caused a slow leakage of intracellular sodium. It is possible that the losses were caused by the presence of potassium or Tris in these reagents, since Slayman and Slayman [16] have reported that these agents cause sodium efflux from wild-type *N. crassa* cells. The shift reagents containing EDTA and EGTA, however, also caused rapid efflux of about 80 and 20% of the sodium content, respectively, when added to large unilamellar vesicles of azolectin containing sodium suggesting that they would not be generally useful for studies in biological systems. These results are tabulated in Table I.

The only shift reagent that proved useful for metabolic studies with the wall-less *N. crassa* cells was $Tm(DOTP)H_2(NH_4)_3$. This reagent resolved the intra-

TABLE I

Properties of shift reagents and effect on N. crassa cells

Shift reagent	Concn. (mM)	Chemical [a] shift	Line [b] width	Reagent stability	*N. crassa* cell damage	% sodium inside liposomes (50 mg/ml)
$Dy(AMPPCP)_2$	5	0	100	+	n.d.	n.d.
$Dy(AMPPNP)_2$	5	5.0	200	+	n.d.	n.d.
$Dy(ATP)_2$	5	5.0	150	−	n.d.	n.d.
$Dy(EDTA)_2$	5	3.0	50	+	yes	2
$Dy(EGTA)_2$	14	2.0	30	+	yes	8.5
$Dy(MDPA)_2$	5	0.35	260	+	n.d.	n.d.
$Dy(PPP)_2$	5	5.5	50	− − −	n.d.	11
$Dy(TETHA)_2$	12	1.5	30	+	yes	<1
Tm(DOTP)	2.5	2.5	35	+	no	n.d.

[a] Difference between shifted and unshifted sodium resonances, ppm.
[b] Line width of shifted external sodium resonance.
n.d., not determined.

and extra-cellular sodium resonances and did not damage the cells. In addition the reagent did not broaden the resonance of the external sodium. The cells remained viable over the course of the NMR measurements as measured by exclusion of trypan blue. Moreover, the areas of the intra and extracellular signals remained constant in aerated cells over the time course of the NMR measurements showing that the intracellular sodium content was stable.

Sodium content in the slime variant of N. crassa

A typical ^{23}Na-NMR spectrum of the *N. crassa* cells in sodium-containing medium using Tm(DOTP) as shift reagent is shown in fig. 1). The average line width of the extracellular signal was 34 Hz and the average line width of the intracellular signal was 53 Hz. When cells were examined at a concentration of 30–50 mg/ml, the intracellular sodium resonance typically represented between 5 and 10% of the total NMR signal. This represents an average intracellular concentration of 11.4 ± 1.5 mM (mean $\pm$ S.E., $N = 7$). When $Dy(AMPPNP)_2$ was used as the shift reagent the average concentration was 11.9 ± 1.2 mM (mean $\pm$ S.E., $N = 2$). These values are in reasonable agreement with the sodium concentration reported by Slayman and Tatum [6] for wild-type *N. crassa* cells grown in Vogel's minimal medium [10] of 14 ± 2 mM.

When the cells were resuspended in MM containing insulin, 100 nM, the intracellular sodium concentration was unchanged compared to that in insulin free medium (11.9 ± 1.4 mM, $N = 6$). When D-glucose, 100 mM, was added to the cells without insulin, the intracellular concentration of sodium initially increased to 14.2 ± 1.0 mM but then decreased slightly over the next 12 min, to 13.0 ± 1.0 mM. When the insulin-treated cells were administered D-glucose the initial intracellular concentration of sodium also increased initially, to 14.8 ± 2.1 mM. In contrast to the case without insulin, the sodium content of these cells continued to increase for the next 12 min, to a calculated value of 16.5 ± 2.2 mM (Fig. 2).

A statistically significant difference was found between the rates of change in intracellular sodium content following glucose addition in the presence and absence of insulin. This rate was calculated separately for each data set (obtained from a single sample) using the method of least squares (Table II). The calculated rates averaged $+1.14 \pm 0.39\%$/min (mean $\pm$ S.E.) in cells pre-treated with insulin, and $-0.63 \pm 0.29\%$/min in control cells (Fig. 2). The difference between these values was significant ($P = 0.005$) using the 't'-test for unpaired variates. The mean values of the sodium content of insulin-treated and control cells at each time point did not differ significantly, however, because of relatively large variations between cell populations run on different days.

When the cells were resuspended and washed in medium where equimolar potassium was substituted for

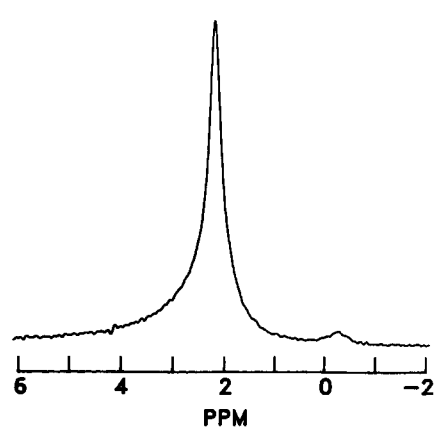

Fig. 1. ^{23}Na-NMR spectra of *N. crassa* cells, 50 mg/ml in MM ([Na] = 25.4 mM) containing Dy(DOTP), 2.5 mM.

TABLE II

Rates of change of intracellular sodium concentration in N. crassa cells following the addition of D-glucose, in presence and absence of insulin

Trial	Rate (%/min)	
	−insulin	+100 nM insulin
1	−1.14	+0.09
2	−0.40	+1.67
3	−0.50	+2.15
4	−1.94	+1.55
5	+0.26	+1.04
6	−0.42	−0.44
7	−0.27	
Average	−0.63 [a]	+1.01 [a]
Standard deviation	0.71	0.89
Standard error	0.29	0.39

[a] The T value for the significance of the difference between the cells treated with insulin and the controls was 3.98 with 11 degrees of freedom. The probability calculated from this T value is 0.0024.

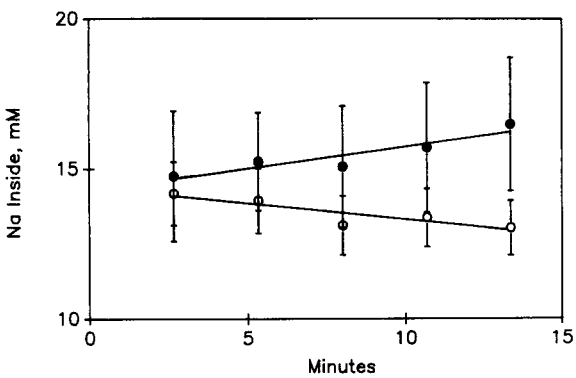

Fig. 2. Effect of insulin on sodium content of *N. crassa* cells. Glucose (100 mM) was added to $t = 0$ after pre-incubation for 15 min in the presence (●——●, $n = 6$) or absence (○——○, $n = 7$) of 100 nM insulin.

sodium, the intracellular sodium was almost completely lost and only a small signal could be detected over the broad NMR signal arising from sodium in the glass NMR tubes. When cells were resuspended and washed in Mes-Tris buffer containing sorbitol, the intracellular sodium content dropped to approx. 5 mM. The signal-to-noise ratio of the intracellular sodium resonance in this buffer was too low to determine the effects of glucose or insulin under these conditions.

Discussion

In order to determine the intracellular sodium content of *Neurospora crassa* slime cells using ^{23}Na-NMR, it was first necessary to identify a suitable shift reagent, i.e., one that remained stable in the presence of secreted proteins, was metabolically inert, did not damage the cells, and gave a suitably large chemical shift. Tm(DOTP) proved to be the only reagent examined that possessed all of these properties (Table I). Using this reagent, the intracellular sodium content of slime cells was measured, and found to be similar to that reported in wild type *N. crassa* cells using a different technique [10].

N. crassa cells, like mammalian cells, excrete sodium and accumulate potassium [6]. In wild-type cells, the sodium and potassium contents appear to mainly be under the control of a potassium/sodium exchanger. Potassium influx is accompanied by efflux of sodium and hydrogen ions during net transport and is an energy-dependent saturable function of the external potassium concentration [16,17]. All three currents (K^+ influx, Na^+ efflux, and H^+ efflux) can be accounted for in terms of a single pump displaying standard Michaelis-Menten kinetics which is distinct from the electrogenic proton translocating ATPase of *N. crassa* [16]. In the absence of potassium the cells take up sodium, which appears to be accompanied by the efflux of protons, however, potassium in the medium inhibits the uptake of sodium [22].

When the cells were washed in buffer in which potassium was substituted for sodium, the cells lost almost all of their internal sodium, consistent with the exchange of sodium for potassium observed by Slayman and Tatum [6] for wild-type cells. In addition, when the cells were washed in Mes-Tris buffer, their intracellular stores dropped to approx. 5 mM. Slayman and Slayman [16] have shown that Tris buffer can result in a rapid reversible loss of both Na^+ and K^+ from wild-type *N. crassa* cells.

When D-glucose was given to the cells in the absence of insulin, the sodium content initially increased, but then decreased. In contrast to *Escherichia coli*, in which addition of D-glucose caused a rapid efflux of intracellular sodium [23] the intracellular sodium content of the fungal cells rose upon initiation of aerobic oxidation of D-glucose. The sodium content of *N. crassa* is in large part regulated by the potassium gradient in the cells, and by the intra and extracellular pH gradient. The pH gradient, which rapidly falls in *E. coli* when nutrients are limiting [23] is remarkably well defended in *N. crassa* cells and is maintained by intracellular buffering in nutrient depleted cell cultures.

In cells treated with insulin, the intracellular sodium content appeared to increase slowly (approx. 0.1 mM/min) following the addition of D-glucose. This is probably not due to a direct effect on the sodium/potassium/proton exchanger, since there is no difference between insulin-treated and untreated cells in the rate of change of intracellular or extracellular pH following addition of glucose [5]. Moreover, sodium transport in *N. crassa* is relatively insensitive to pH changes in these ranges [17,22]. It is possible that the very small net change in sodium content reflects cation influx accompanying changes in transport of phosphate or nitrate, or reflects changes in intracellular cations

necessary to neutralize acidic metabolites produced following the addition of D-glucose to the cells. When D-glucose is administered to slime, the cells rapidly produce gluconate and alanine. Gluconate appears in both cells and medium, as a steady-state intermediate, at a concentration of approx. 1–2 mM. L-Alanine accumulates as an intracellular metabolic end product at a rate of approx. 0.3 mM/min in control cells and 0.4 mM/min in insulin-treated cells [5]. Carbon dioxide is also rapidly produced by slime cells and the rate of its production is also 10–20% higher in insulin-treated cells [4]. It is conceivable that the small differences in intracellular sodium content between insulin-treated cells and controls reflect these metabolic differences.

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