Vol. 95, No. 1, 1980
July 16, 1980

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 73-81

PHOSPHOCREATINE IN EHRLICH ASCITES TUMOR CELLS DETECTED BY NONINVASIVE ^{31}P NMR SPECTROSCOPY

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Received May 19,1980

Summary: ^{31}P NMR spectra of intact Ehrlich ascites tumor cells of high phosphorylation potential reveal a well-defined resonance peak assignable to phosphocreatine, corresponding to 2.3 μmoles/ml cell H_2O in adenosine-treated cells containing 5.2 μmoles ATP/ml. The NMR spectrum of Ehrlich cells incubated with iodoacetate and glucose indicates depletion of phosphocreatine and ATP to undetectable levels and substantial accumulation of fructose-1,6-bisphosphate. From the difference between the chemical shifts of internal P_i and phosphocreatine resonances, the intracellular pH was estimated to be 7.1 ± 0.1 in protein-synthesizing cells suspended in a medium of pH 7.4 at 10°C. Ehrlich cells are unable to transfer the labeled amidine group from L-(guanidino-^{14}C)-arginine to the large intracellular glycine pool to form labeled guanidinoacetate, the demethylated precursor of creatine. These results imply that the synthesis of phosphocreatine in ATP-rich Ehrlich cells is limited primarily by the extracellular free creatine supply, the extent of which depends upon the degree of cachectic perturbation of energy and nitrogen metabolism of the tumor-bearing host.

INTRODUCTION

The synthesis of phosphocreatine is catalyzed by only one known enzyme, creatine kinase (EC 2.7.3.2) (1). The creatine kinase reaction is usually considered to have a major physiological role only in muscle and brain, because these tissues contain a large dynamic reservoir of phosphocreatine. Significant activities of creatine kinase, however, have been reported (2,3) to be present in many other vertebrate tissues and cells. Creatine kinase has recently been shown to be retained in substantial activity in murine tumors (4), and its brain-type BB isozyme has been proposed as a new human tumor-associated marker enzyme (5).

In mouse Ehrlich ascites tumor cells, creatine kinase consists exclusively of the BB isozyme and has appreciable activity, which is 25-fold higher than that of adenylate kinase (4). The reported failure to detect phosphocreatine in Ehrlich cells by ^{31}P NMR (6) apparently is not due to inadequate kinase

0006-291X/80/130073-09$01.00/0

activity. As part of our continuing noninvasive ^{31}P NMR studies of phosphorus-containing metabolites and their Mg^{2+} complexation in Ehrlich cells (7), we report here that, contrary to the above mentioned observation (6), a distinct single resonance peak, characteristic of phosphocreatine, is observed in the NMR spectra of intact Ehrlich cells. Phosphocreatine concentrations approach that of ATP in cells of high phosphorylation potential and decrease beyond detection upon depletion of ATP in iodoacetate-treated glycolyzing cells. The noninvasive ^{31}P NMR technique was also applied to the estimation of intracellular pH from the separation between the chemical shifts of intracellular P_i and phosphocreatine resonances.

Since L-arginine, one of the three amino acids required for creatine biosynthesis, was previously shown to be present in Ehrlich cells at extremely low levels (8), the possibility that cellular deficiency of free arginine was partly due to its conversion to a creatine precursor was considered. Here we report that Ehrlich cells are unable to transfer the amidine moiety from L-(guanidino-^{14}C)arginine to the sizable cellular glycine pool to form labeled guanidinoacetate, the demethylated precursor of creatine. The ability of Ehrlich cells to synthesize phosphocreatine, therefore, depends upon an extracellular source of free creatine and its transport into the cells against a concentration gradient.

MATERIALS AND METHODS

Ehrlich ascites carcinoma cells were propagated in female ICR mice as previously described (9). Mice were fed Emery Morse Diet 911R, mainly composed of plant products and 20% by weight of dried skim milk (cow's milk contains 0.3 mM creatine) (10). Ehrlich cells were prepared for *in vitro* incubation and subsequently for NMR measurement as indicated previously (7), under optimal conditions for maintaining ATP at a high level and thus preventing the degradation of any phosphocreatine initially present. Noncellular control experiments with phosphocreatine, ATP, MgATP, and fructose 1,6-bisphosphate were performed in 20 mM bis-tris buffer, pH 7.2, at 0.15 M ionic strength. ^{31}P NMR spectra of intact cells were recorded at 40.5 MHz with a Varian XL-100 spectrometer in the Fourier transform mode (11) employing wide-band proton noise decoupling. Each sample contained 10% D_2O to allow heteronuclear field frequency locking and a 50% suspension of Ehrlich cells in a total volume of 1.4 ml was used for NMR studies under previously stated conditions (7). Protein-free cell extracts were prepared in 0.25 N perchloric acid at 0° and were neutralized without delay to pH 7.2 with KOH containing potassium glycylglycinate (9), to minimize hydrolysis of phospho-

creatine (3). NMR spectra of the neutralized cell extracts were obtained at a frequency of 145.7 MHz with a Bruker HX360 spectrometer operating in the Fourier transform mode.

For the arginine-glycine transamidination experiments, 5% Ehrlich cell suspensions containing cellular protein labeled with 4 μcuries of L-(guanidino-^{14}C)arginine (New England Nuclear, 26 mCi/mmol) during prior incubation were incubated at 37.5° for 2 h in the presence of 0.2 mM cycloheximide to inhibit protein synthesis with or without 3 mM guanidinoacetate. Protein-free cell extracts were applied to the dual columns of the Beckman model 120 amino acid analyser and were analyzed for 3 types of reactive components: 1) ninhydrin-reactive compounds (12), 2) guanidinium compounds in eluted fractions by a modified Sakaguchi color reaction (13), and 3) ^{14}C radioactivity in fractions eluted with citrate buffer and determined by means of a Beckman LS-250 scintillation counter employing Hydromix (Yorktown) scintillation solution.

RESULTS AND DISCUSSION

The ^{31}P NMR spectrum of Ehrlich ascites tumor cells incubated under conditions permitting rapid protein synthesis shows seven distinct peaks including six which were previously identified (6) as fructose-1,6-bisphosphate, phosphorylcholine, P_i, and the three phosphoryl groups of ATP. In contrast to the previous report (6), we found a seventh prominent resonance peak at -3.1 ppm (see Fig. 1a) to be consistently present in spectra of Ehrlich cells of high phosphorylation potential. This resonance peak was also found in neutralized perchloric acid extracts of Ehrlich cells containing normal and elevated ATP levels (Fig. 2a and 2b) and is assignable only to phosphocreatine on the basis of noncellular controls. This chemical shift was also previously assigned by others to phosphocreatine in studies of intact HeLa cells (14), rat brain (15), and skeletal muscles (16,17).

The improved resolution of our spectra of Ehrlich cells in figure 1, compared to previous studies (6), must arise from the use of lower magnetic field (23.5 kilogauss) in our study. At the higher magnetic field used in previous studies (85 kilogauss), the chemical shift anisotropy contribution to the ^{31}P linewidths apparently broadens the observed lines quite significantly. Since this broadening is not observed with cell extracts, it must arise from longer rotational correlation times of intracellular metabolites in intact cells. Whether this altered correlation time originates from the

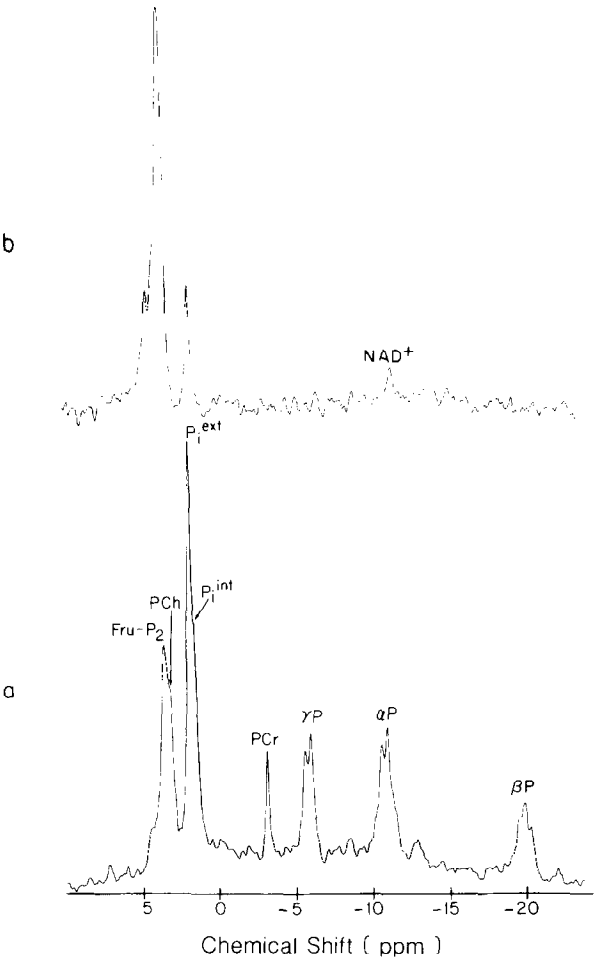

Fig. 1. ^{31}P NMR spectra at 40.5 MHz of a 50% suspension of Ehrlich ascites tumor cells recorded at 10°. Spectra are referenced to 85% phosphoric acid at 0 ppm. (a) Cells after incubation at 37.5° for 30 min in an isotonic salt solution containing 1 mM adenosine, 3 mM glucose, 3 mM P_i, 1.5 mM $MgSO_4$, 6 mM KCl, 30 mM Hepes buffer, pH 7.4, and a mixture of essential amino acids. (b) Cells incubated with 1 mM iodoacetate in addition to the constituents listed above. For obtaining NMR spectra, an acquisition time of 1.6 sec, a spectral width of 2500 Hz and an exponential filtering time constant of 0.1 sec were used. The length of the free induction signal was doubled by zero-filling prior to Fourier transformation to obtain a digital resolution of 0.31 Hz. Pulses (~90°) were recycled without any additional time-delay for magnetization recovery. Two thousand pulses of NMR signal were time-averaged to obtain the spectra shown. Fru-P_2, fructose-1,6-bisphosphate; PCh, phosphorylcholine; P_i^{ext}, extracellular P_i; P_i^{int}, intracellular P_i; PCr, phosphocreatine; γP, αP, and βP, resonances of the phosphoryl groups of ATP; NAD^+, nicotinamide adenine dinucleotide.

binding of phosphometabolites to cellular macromolecular components or is due to higher viscosity of the cytoplasm remains to be investigated.

The cellular level of phosphocreatine was estimated from the relative areas under the peaks of phosphocreatine and βP of ATP (Fig. 1a) and from the absolute

ATP concentration determined enzymatically using hexokinase and glucose-6P dehydrogenase (9). The concentrations of phosphocreatine and ATP were 2.3 and 5.2 μmoles/ml cell H_2O, respectively. That our determination of concentration of phosphocreatine is free of ^{31}P saturation effects is indicated by the similarity of values obtained with 0.8 and 1.6 sec pulse recycle times. In previous studies (18), ascites tumor cells were found to contain ∿2 mM acid-labile phosphate, in addition to the phosphates of ADP and ATP, that was readily available for the intracellular phosphorylation of 2-deoxyglucose by hexokinase. The present investigations suggest that phosphocreatine is the previously unidentified acid-labile ascites tumor component that is rapidly depleted along with ATP. The NMR

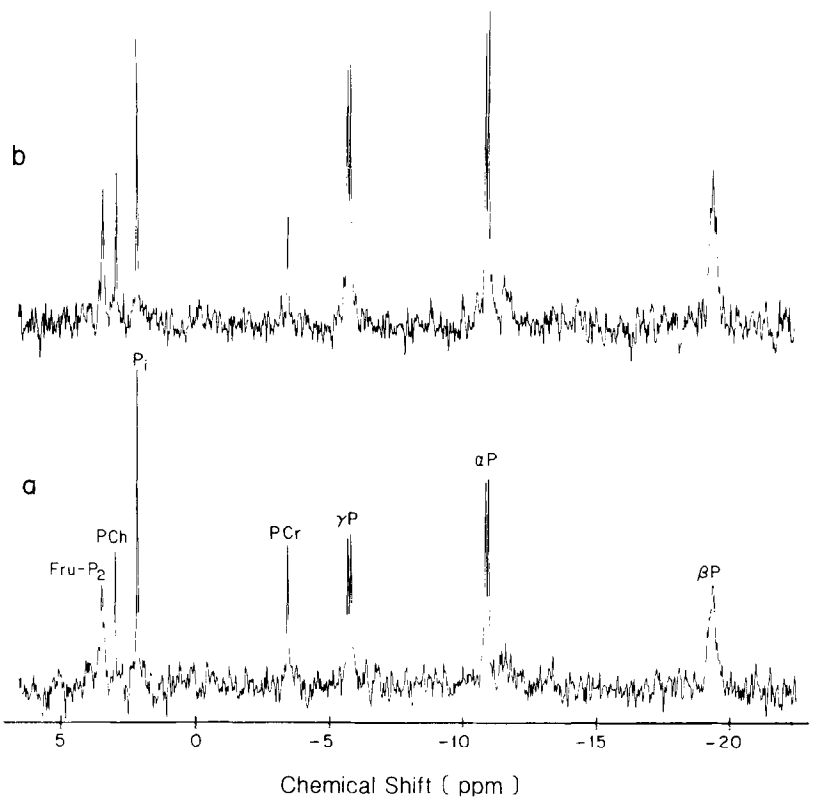

Fig. 2. ^{31}P NMR spectra at 145.7 MHz of neutralized perchloric acid (0.25 N) extracts of Ehrlich ascites cells. (a) Cells incubated without adenosine, otherwise as in Fig. 1a. (b) Cells incubated with adenosine and other constituents as in Fig. 1a. For obtaining NMR spectra, an acquisition time of 1.6 sec, a spectral width of 5000 Hz and an exponential filtering time constant of 0.3 sec were used. Pulses (∿60°) were recycled with an additional time-delay of 12 sec for magnetization recovery. Two hundred pulses of NMR signal were time-averaged to obtain the spectra shown.

spectrum of Ehrlich cells incubated with iodoacetate and glucose (see Fig. 1b) indicates that both phosphocreatine and ATP decreased to undetectable levels, NAD^+ remained detectable, most of the P_i disappeared, while fructose-1,6-bisphosphate increased substantially, as would be expected from inhibition of the glycolytic pathway at the glyceraldehyde phosphate dehydrogenase step by iodoacetate. The phosphocreatine pool in the tumor cells can be expected to maintain the ATP concentration for only ∿15 sec, if other ATP biosynthesis pathways are completely inhibited (19). Since phosphocreatine was found to inhibit phosphofructokinase at pH 7.4 nearly 50% (20) within the range of the intracellular phosphocreatine levels reported here, it is possible that this phosphagen may have a role in the regulation of Ehrlich cell glycolysis. Although phosphocreatine was also reported to inhibit other glycolytic enzymes (1), the presence of contaminating inhibitors in phosphocreatine samples used in those studies suggests that the regulatory role of phosphocreatine requires reinvestigation (21,22). When Ehrlich cells were incubated with adenosine to increase the MgATP/MgADP ratio almost 4-fold as was previously reported (7,9), the concentration of phosphocreatine did not increase (see Fig. 2b). This anomalous response of phosphocreatine to an increase in phosphorylation potential may be due to other induced changes in intracellular conditions, such as a decrease in pH, which would shift the creatine kinase equilibrium (23) toward a lower phosphocreatine/creatine ratio.

The intracellular pH can be estimated by noninvasive ^{31}P NMR spectroscopy from the separation between phosphocreatine and the internal P_i peaks (24). This separation for Ehrlich cells (see Fig. 1a, shoulder is peak P_i^{int}) is 195 $\pm$ 2 Hz, corresponding to an internal pH of 7.1 $\pm$ 0.1. The difference between the chemical shifts of external P_i and phosphocreatine of 207 $\pm$ 2 Hz corresponds to an external pH of 7.4 $\pm$ 0.1. This pH gradient of -0.3 across the Ehrlich cell membrane is essentially in agreement with the findings previously reported in extensive pH studies based on the distribution ratio of a weak acid across the cell membrane (25).

Table I

Lack of formation of labeled guanidinoacetate in Ehrlich cells by transamidination with an expanded pool of free (guanidino-^{14}C)arginine

Eluted Component	Radioactivity (dpm/ml cell H_2O) No Incubation	2h Incubation with CHI	2h Incubation with GA, CHI
Urea	2,880	19,900	16,300
Arginine	2,300	26,800	28,900
GA	~5	~50	0

Ehrlich cells were previously incubated for 1 h with (guanidino-^{14}C)arginine (see Methods section) in addition to the medium constituents indicated in Fig. 1a. The cell proteins were thus labeled with 3.7 x 10^6 dpm/ml packed cells. Washed cells were then incubated in the presence of 0.2 mM cycloheximide (CHI) with or without 3 mM guanidinoacetate (GA) for 2h, and the reaction was stopped by 0.25 M perchloric acid. Components of the acid-soluble fraction were separated and analyzed as indicated in the Methods section. GA identified by the modified Sakaguchi method had elution times midway between those of alanine and valine.

We have previously shown (7) that in Ehrlich cells with a free Mg^{2+} level of 0.44 mM, 88% of the total ATP and ~50% of the total ADP is complexed with Mg^{2+}. From the dissociation constant for Mg-phosphocreatine (K_D^{MgPCr} = 25 mM) (23), the above cellular free Mg^{2+} value, and the equation, $[PCr]_T/[MgPCr] = 1 + K_D/[Mg^{2+}]_f$, we calculate that only ~2% of the total phosphocreatine is present as the Mg^{2+} complex. If in Ehrlich cells, the creatine kinase reaction were at the equilibrium defined by the equation ($K_{eq} = [PCr][MgADP][H^+]/[Cr][MgATP]$), the intracellular creatine level can be calculated to be ~12 mM, where the phosphocreatine concentration is taken to be 2.3 mM, the MgATP/MgADP ratio is 38 (7), and $K_{eq} = 3.08 \times 10^{-10}$ M at pH 7.2 (23). Assuming that this ratio of [Cr]/[PCr] = ~5 at pH 7.2 applies to the Ehrlich cells reported to contain 2 µmoles total creatine/ml packed cells (26), the calculated level of 0.4 µmole phosphocreatine/ml cell H_2O may have been within the limits of detection of previous ^{31}P NMR studies (6).

As indicated by the data in Table I, Ehrlich cells apparently are not able to transfer the labeled amidine group from (guanidino-^{14}C)arginine to the ~6 mM

free glycine pool or to exchange it with excess added guanidinoacetate to form detectable labeled guanidinoacetate. Instead, arginine is preferentially metabolized to urea and ornithine, particularly when the free arginine pool is expanded by cycloheximide inhibition of protein synthesis (8). Furthermore, Ehrlich cells incubated with L-(^{3}H)ornithine do not form any detectable labeled arginine (Yushok, W.D. and Leary, R., unpublished data). This is an additional indication of a lack of a functional amidine exchange system (1). Since biosynthesis of guanidinoacetate is considered to be rate limiting for creatine synthesis (1), Ehrlich cells apparently obtained creatine for phosphocreatine synthesis from an extracellular source and can transport it against a concentration gradient to allow the creatine kinase reaction at near equilibrium to form significant levels of phosphocreatine. The release of free creatine into body fluids of the tumor-bearing host from muscle tissues may be the result of a metabolic shift to a negative nitrogen and energy balance induced by the stringent nutritional demands of rapid uncontrolled proliferation of cancer cells. The nature and extent of these cachectic metabolic perturbations of neoplastic disease remain to be investigated.

ACKNOWLEDGEMENTS

This work was supported by the National Institutes of Health grants AM-19454 and CA-12650, by grants CA-06927 and RR-05539 to this Institute from the National Institutes of Health and by an appropriation from the Commonwealth of Pennsylvania. R.K.G. is a Research Career Development Awardee of the United States Public Health Service (AM(NIH)-00231). The 145.7 MHz studies were done at the Middle Atlantic Regional NMR Facility at the University of Pennsylvania supported by NIH Grant RR-542.

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