^{31}P-nuclear magnetic resonance studies of intact plasmodia of *Physarum polycephalum*

Kazuhiro Kohama, Masaru Tanokura* and Kazuhiro Yamada*

Department of Pharmacology, Faculty of Medicine, University of Tokyo, Bunkyo-ku, Tokyo 113 and **Department of Physiology, Medical College of Oita, Oita 879-56, Japan*

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^{31}P-nuclear magnetic resonance spectra were obtained from intact plasmodial cells of *Physarum polycephalum*, where cytoplasmic streaming is generated by actin–myosin–ATP interaction. Several peaks were resolved and identified. They included ATP, ADP, orthophosphate and polyphosphates. Peaks for phosphocreatine, phosphoarginine or AMP were not detected. The intracellular pH and concentrations of ATP and free Mg^{2+} were estimated to be pH 6.9, 0.2–0.5 mM, and about 1 mM, respectively.

^{31}P-NMR *Intracellular pH* *ATP* *Mg^{2+}* Physarum polycephalum *Actomyosin*

1. INTRODUCTION

The plasmodium of the slime mold, *Physarum polycephalum* shows vigorous shuttles of cytoplasmic streaming. Actin–myosin–ATP interaction has been proposed to generate the motive force for streaming in a similar way to the interaction causing contraction of skeletal muscle [1] since an actomyosin-like protein was prepared from plasmodia [2].

However, the metabolism of phosphorus compounds, including ATP, is quite different between plasmodium and skeletal muscle. For example, the ATP concentration in plasmodium is one-fifth (or less) of that in skeletal muscle [3,4]. Moreover, phosphocreatine and phosphoarginine are absent in plasmodium [3], and polyphosphate has been proposed as a phosphagen [5]. These observations are made on the extract of plasmodia, which raises the question whether the difference is a product of the extraction procedures. Studies using ^{31}P-nuclear magnetic resonance (^{31}P-NMR) of living plasmodia may well answer the question, because the phosphate compounds are measured in a non-destructive way [6].

Here, we subject intact plasmodial cells to ^{31}P-NMR measurement to estimate intracellular concentrations of phosphate compounds. From the ^{31}P-NMR spectrum, we also estimated intracellular pH and Mg^{2+} concentrations, which affect actin–myosin–ATP interaction. In the light of the estimated concentrations, the physiological roles of the inhibitory effect of Ca^{2+} on actin–myosin–ATP interaction [7–10] in regulating the cytoplasmic streaming is discussed.

2. MATERIALS AND METHODS

Plasmodia of the Colonia isolate of *Physarum polycephalum* [11] were cultured in the axemic medium described in [12] with slight modifications; the concentration of KH_2PO_4 was reduced to 0.2 g/l. After 3–4 days shaking at 25°C, the culture reached a stationary phase. The plasmodial cells were precipitated either by standing for 5–10 min or by centrifuging at 900 × *g* for 1–3 min. The precipitated cells were put into a 25-mm diameter glass sample tube for ^{31}P-NMR measurement.

The ^{31}P-NMR spectra were recorded at 81 MHz on a Bruker WM 200 wb wide-bore spectrometer with 5 kHz spectral width, 60 pulse width and 2 s recycling time [13,14]. The probe temperature was

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kept at 22°C throughout the measurements. Each spectrum was obtained as a time average of 1024 or 2048 transients.

3. RESULTS

Fig.1 shows a typical ^{31}P-NMR spectrum of living plasmodia of *Physarum polycephalum*. We assigned the peaks of the spectrum to phosphate compounds according to their chemical shifts listed in [6].

Peaks 3 and 4 resonating at 1.75 ppm and 0.84 ppm, respectively, were identified as orthophosphate peaks (fig.1). As the orthophosphate in the culture medium (pH 4.5) resonated at 0.84 ppm (not shown), peaks 3 and 4 were the resonances arising from intracellular and extracellular phosphates, respectively.

Intracellular pH values (pH_i) were calculated from the chemical shift of orthophosphate (δ^{obs}) according to the following equation:

$$pH_i = pK_a + \log (\delta^{obs} - \delta_a)/(\delta_b - \delta^{obs})$$

where δ_a and δ_b are the chemical shifts of $H_2PO_4^-$ (δ_a = 0.85 ppm) and HPO_4^{2-} (δ_a = 3.31 ppm), respectively [15]. A pK_a value of 6.98 was calculated for an ionic strength of 0.03 in plasmodia [16] at 22°C, using the constant and the equations developed in [17].

The chemical shift of peak 3 was shifted to higher field as the ^{31}P-NMR measurement proceeded (fig.2); pH_i decreased from 6.81 to 6.69 during the initial 2.5 h (fig.3). We considered the pH_i of healthy plasmodial cells to be pH 6.9 by extrapolation to zero time (fig.3). If we assume that

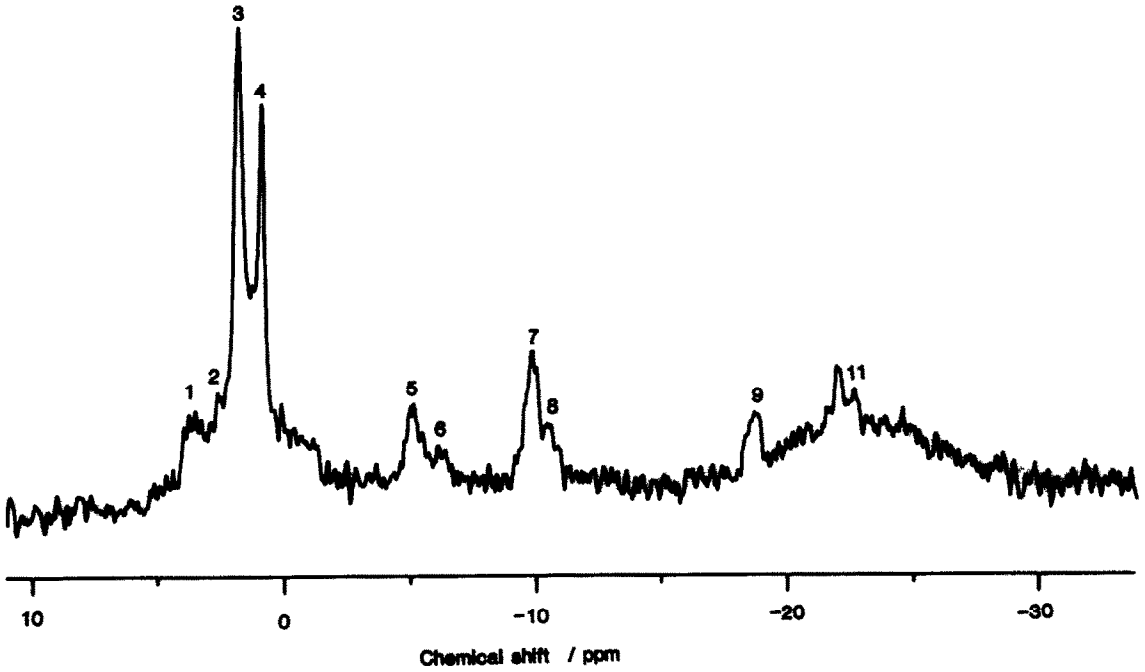

Fig. 1. ^{31}P-NMR spectrum of plasmodial cells of *Physarum polycephalum* recorded during the initial 1.2 h. Chemical shifts are expressed in ppm down-field from 85% phosphoric acid. Peak 1 (3.65 ppm) and peak 2 (2.56 ppm), sugar phosphates; peak 3 (1.75 ppm), intracellular orthophosphate; peak 4 (0.84 ppm), extracellular orthophosphate; peak 5 (−5.09 ppm) and/or peak 6 (−6.27 ppm), γ-phosphate of ATP + β-phosphate of ADP; peak 7 (−9.98 ppm), α-phosphates of ATP and ADP; peak 8 (−10.55 ppm) NAD and/or NADH; peak 9 (−10.55 ppm), β-phosphate of ATP; peak 10 (−22.09 ppm) intracellular polyphosphate; peak 11 (−22.75 ppm) extracellular polyphosphate. The peaks were mostly assigned according to Bárány and Glonek [10] under the assumption that the contributions NTP and NDP other than ATP and ADP were negligibly small. N.B., resonances for phosphocreatine, phosphoarginine, and AMP were not identified as major peaks.

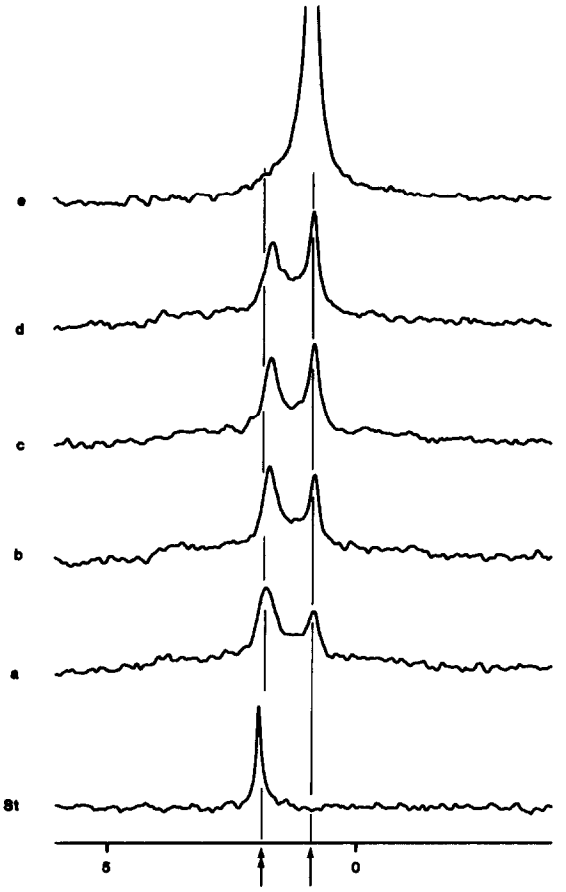

Fig. 2. Orthophosphate region of ^{31}P-NMR of plasmodial cells of *Physarum polycephalum*. ^{31}P-NMR spectra were collected during successive 0.62 h periods. Vertical bars with single and double arrows were drawn at 0.84 ppm (peak 4) and 1.75 ppm (peak 3), respectively. (a) 0–0.62 h; (b) 0.62–1.2 h; (c) 1.2–1.9 h; (d) 1.9–2.5 h; (e) 7.7–9.3 h; St, resonance from 1 mM KH_2PO_4 in 30 mM KCl at pH 7.0.

the pH gradient across the membrane of plasmodial cells is maintained by a proton-pump [18], an intracellular acidification might be caused by reduction in the activity of the proton-pump. Our estimated value of pH_i is lower than that obtained in [19] (pH 7.0–7.5), but higher than that obtained in [20] (pH 5.8–6.6).

The intracellular concentration of orthophosphate during the initial 0.62 h (a in fig.2) was estimated at 1.7 mM by comparison with the integrated area of the 1 mM phosphate peak (St, in fig.2). As the extracellular space of the packed cells

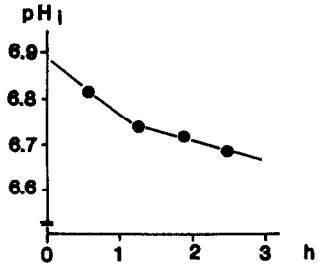

Fig. 3. Time course of the changes in the intracellular pH. Using spectra in fig.2, intracellular pH (pH_i) was calculated from the equation in the text. The pH_i extrapolated to zero time was considered as the pH_i of healthy plasmodial cells.

in the sample tube was estimated at 53% from the area of peak 4 (a in fig.2), the intracellular concentration of orthophosphate was estimated at 3.2 mM. Plasmodial cells were grown in a culture medium containing 1.5 mM phosphate, suggesting that the cells may have a phosphate uptake system. intracellular orthophosphate concentration decreased during ^{31}P-NMR measurements, as shown by the decrease in the integrated area of peak 3 (fig.2). The decrease is paralleled by a decrease in pH_i, suggesting that the uptake of orthophosphate may be associated with the proton-pump.

We compared the integrated area of β-phosphate of ATP (peak 9, fig.1) with measurements of a known ATP concentration under similar conditions, i.e., 0.5 mM Mg-ATP, 1 mM Mg^{2+} and 30 mM KCl at pH 7.0. The area of peak 9 corresponded to 0.2 mM ATP. Taking the value of 53% for the extracellular space in the sample tube, the intracellular concentration of ATP was calculated to be 0.4 mM. Similar determinations in other experiments ranged from 0.2 to 0.5 mM. Our estimated value is consistent with the total cytoplasmic ATP concentration in the homogenate of plasmodial cells [3,4]. It should be noted that the ATP concentration decreased during the course of experiments lasting for many hours (not shown). However, during the initial 1.2 h after starting the ^{31}P-NMR measurement, the ATP concentration was almost constant (not shown). As the plasmodial cells were packed into the sample tube for the ^{31}P-NMR measurement, they may have suffered from anoxia during prolonged measurement. The anoxia may also be related to the reduction in

pH_i (fig.3) and in intracellular orthophosphate concentration (fig.2) as mentioned above.

The intracellular free $[Mg^{2+}]$ was estimated from the following equations [21]:

$$[Mg^{2+}] = K_d\,(1-\phi)/\phi$$

$$\phi = (\delta - \delta^{Mg\text{-}ATP})/(\delta^{ATP} - \delta^{Mg\text{-}ATP})$$

where δ is the measured separation between the α- and β-phosphate resonances of ATP; $\delta^{Mg\text{-}ATP}$ (8.51 ppm) and δ^{ATp} (10.96 ppm) are values of δ in the presence of a saturating level of Mg^{2+} and in the absence of Mg^{2+}, respectively, under intracellular conditions. A K_d value of 90 μM was obtained with 30 mM K^+ [16], and pH 6.9 from constants described in [22]; ϕ is the ratio of concentrations of free ATP to total ATP; most (90%) of the intracellular ATP is present in the form of Mg-ATP (fig.1). The ^{31}P-NMR spectrum shown in fig.1 gave an estimated value for intracellular $[Mg^{2+}]$ of 0.78 mM. The estimated value varied between 0.78 and 1.67 among different ^{31}P-NMR measurements. The intracellular $[Mg^{2+}]$ is therefore thought to be about 1 mM.

4. DISCUSSION

In the present study, intracellular pH and concentrations of ATP and free Mg^{2+} in living plasmodia of *Physarum polycephalum* are estimated at 6.9, 0.2–0.5 mM and about 1 mM, respectively. Most of the intracellular ATP appears to bind Mg^{2+}.

As the previous conditions, when examining the effect of Ca^{2+} on actin–myosin–ATP interaction [7–10], were rather arbitrary, the effect was reexamined in the light of the estimated concentrations. In 30 mM KCl [16], 1 mM Mg^{2+} and 0.5 mM Mg-ATP at pH 6.9, ATPase activity of *Physarum* myosin in the presence of skeletal muscle actin was the highest (100%) in the presence of EGTA. The activity was reduced to 50% by 2 μM Ca^{2+}, to 20% by 10 μM Ca^{2+} and to 0% by 50 μM Ca^{2+} (unpublished). This observation suggests that the myosin-linked inhibitory effect of Ca^{2+} on actin–myosin–ATP interaction works in living plasmodia.

The integrated area of peak 7 (α-phosphates of ATP and ADP) was 1.8-fold larger than the area of peak 9 (β-phosphate of ATP) (fig.1), indicating that the intracellular ADP concentration was fairly high, i.e., about 80% of ATP concentration. This is consistent with the previous report [3]. ADP increased interaction between *Physarum* actin and myosin as measured by the superprecipitation [7]. The physiological role of intracellular ADP remains to be established.

Phosphoruses of AMP, phosphocreatine and phosphoarginine resonate at 3.7, −2.3 and −3.5 ppm [6], respectively. We were not able to detect these peaks in the spectrum shown in fig.1, indicating that intracellular concentrations of these compounds are negligibly small. The absence of phosphocreatine and phosphoarginine in *Physarum* plasmodia confirms previous reports [3,5]. Absence of AMP suggests that myokinase activity, which is present in natural actomyosin preparations [7,23], may be low in the living plasmodia and that ATP pyrophosphohydrolase, whose activity is very high in the extract of plasmodial cells [24], may exist in an inactive form in the living plasmodia.

Since non-terminal phosphate of polyphosphate in the extract of *Physarum* plasmodia resonated at 21.82 ppm at pH 7.0 (about the intracellular pH) and at 22.24 ppm at pH 4.4 (about the extracellular pH) (unpublished), peak 10 and peak 11 (fig.1) were identified as polyphosphate peaks present in intracellular and extracellular compartments, respectively. The ^{31}P-NMR spectrum of the culture medium after sedimenting plasmodial cells showed a fairly high peak of polyphosphate, whereas the intracellular polyphosphate peak (peak 10) was often undetectable (unpublished). To examine whether polyphosphate works as a phosphagen instead of phosphocreatine or phosphoarginine [5], changes in intracellular polyphosphate must be followed under various culture conditions.

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