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Original Contribution

HIGH EXTRACELLULAR INORGANIC PHOSPHATE CAUSES REDUCTION OF INTRACELLULAR FREE MAGNESIUM IN PERFUSED RAT HEART

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ABSTRACT

The aim of this study was to measure the concentration of intracellular free magnesium (free $[Mg^{2+}]i$) in isolated rat heart by analyzing ^{31}P NMR spectra obtained from the linear prediction z-transform method (LPZAR), and to investigate the effect of extracellular inorganic phosphate ([Pi]o) on myocardial Mg metabolism. Free $[Mg^{2+}]i$ of Langendorff perfused hearts was assessed by ^{31}P NMR spectroscopy using LPZAR. The ionic environment was altered by changing [Pi]o from 1.2 (physiological concentration) to 5.0 mM in Kebs-Henseleit buffer during the experiment and changes in free $[Mg^{2+}]i$ were calculated from the frequency difference between α- and β-ATP in the ^{31}P NMR spectrum. The spectra obtained using LPZAR provided ATP peaks sharp enough to calculate chemical shift of β-peak of ATP which is used to calculate the change of free $[Mg^{2+}]i$. When [Pi]o was increased from 1.2 mM to 5.0 mM and then, restored to 1.2 mM, free $[Mg^{2+}]i$ decreased significantly from 0.53 ± 0.05 mM to 0.407 ± 0.025 mM and returned to the control level of 0.60 ± 0.05 mM: alteration of [Pi]o inversely affected the free $[Mg^{2+}]i$. These results suggest that the movement of free Mg^{2+} involves Mg^{2+}-Pi exchange mechanism across the plasma membrane in rat heart.

Key words: Intracellular free magnesium, 31p NMR spectroscopy, linear prediction z-transform, rat heart, Mg^{2+}/Pi exchanger

INTRODUCTION

A growing body of evidence indicates that Mg^{2+} plays an important role in regulating a variety of cardiac cell functions such as ion channel activities and Ca^{2+} release from the sarcoplasmic reticulum (1, 2, 3, 4). However, the mechanisms of Mg^{2+} mobilization to and from the cells including Mg^{2+} transport pathways are not well defined. In 1986, Fry (5) proposed a Na^+/Mg^{2+} exchanger in the plasma membrane using Na^+-selective and Mg^{2+}-selective electrodes. Though a similar Mg^{2+} transport system has been described (6), some study failed to support the evidence that such a system exists in the plasma membrane (7). The reason for the discrepancy was attributed to methodological artifact caused by nonspecific response of the Mg^{2+}- selective electrode to other ions such as Na^+ and K^+ (5, 8). The other evidence which demonstrated the modulation of Mg^{2+} by Ca^{2+} and H^+ using Mg^{2+}-sensitive dye (9) was also questioned since the Mg^{2+} dye was shown to have a significant interference with Ca^{2+} and H^+ (8, 10). Therefore, the details of Mg^{2+} homeostasis remains to be elucidated.

In our previous study (11), it was shown that the concentration of intracellular free magnesium (free $[Mg^{2+}]i$) decreases when extracellular inorganic phosphate (Pi) was raised: suggesting the chemical and electrical energy-dependent exchange between Mg^{2+} and Pi. In that experiment the $[Mg^{2+}]i$ was indirectly estimated from the measured citrate/isocitrate ratio and the intracellular pH using the perfused rat preparation. Thus, the present study was aimed to investigate the influence of extracellular Pi on the myocardial Mg^{2+} metabolism using a more direct approach, the ^{31}P NMR method. Measurement of $[Mg^{2+}]i$ by the ^{31}P NMR method is based on the equilibrium between the free and complex form of ATP. It is not interfered with

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Na^+, K^+, Ca^{2+} or H^+ ions, unlike Mg^{2+}-electrodes or Mg^{2+}-sensitive dyes (12, 13, 14). However, the spectra obtained need to be analyzed with caution since small difference of chemical shift observed in ATP results in large difference in Mg^{2+} value which is calculated by the K_α for Mg^{2+}ATP. Therefore upon analyzing the ^{31}P NMR spectra we used the linear prediction z-transform (LPZAR) method along with the standard fast Fourier transform (FFT) method to overcome that methodological limitation. LPZAR method can provide significantly better spectra both in sensitivity and resolution than the FFT method (15,16).

MATERIALS AND METHODS

Animals and anesthesia

Healthy ad libitum fed Wistar rats (Japan SLC Inc. Shizuoka, Japan) weighing 400-500 g were anesthetized by an i.p. injection of sodium pentobarbita1 (50 mg/kg body weight) and heparinized. The investigation Was Performed in accordance with the Guidelines for animal experimentation in School of Medicine, Tokai University and the aide for the care and use of laboratory animals published by the US National Institutes of Health (NIH publication No. 85-23).

Heart perfusion

The heart was removed and perfused using the Langendorff apparatus with a non-circulating modified Kebs-Henseleit buffer which contained 117 mM NaCl, 25 mM $NaHCO_3$, 5.9 mM KCl, l.2 mM NaH_2PO_4, 1.1 mM $CaCl_2$, 0.52 mM $MgCl_2$, and 10 mM glucose for a control perfusion period. The buffer was continuously aerated with a 95% O_2, 5% CO_2 gas mixture and maintained at a pH of 7.4. The coronary perfusion pressure was adjusted at 70 mmHg and then the heart was placed in a l5 mm diameter plastic tube surrounded by solenoid coil. A surface of the effluent from perfused heart in the plastic tube was adjusted to the level of the root of the aorta and an overflow of the effluent was continuously withdrawn through an aspirator to keep its surface constant. The tube was then inserted into the NMR bore. Reservoirs, perfusion lines, perfusion apparatus and the NMR probe were maintained at 37 ^oC during the entire experiment. Heart rate and left ventricular pressure were continuously monitored. The time from anesthetizing the rat to stabilizing the heart in the NMR spectrometer was about 15 min. Four hearts were subjected to high Mg^{2+} perfusion and 6 hearts were subjected to high Pi perfusion followed by control perfusion.

EXPERIMENTAL PROTOCOL

High Mg^{2+} perfusion; after a 10 min equilibration period with control buffer, ^{31}P NMR spectra were collected for the next 30 min and then the buffer was switched to high Mg^{2+} buffer which contained 20 mM $MgCl_2$. After a 30 min equilibration period with high Mg^{2+} buffer, ^{31}P NMR spectra were collected for 45 min.

High Pi perfusion; after a 10 min equilibration period with control buffer, ^{31}P NMR spectra were collected for the next 30 min and then the buffer was switched to high Pi buffer which comprised 114 mM NaCl, 25 mM $NaHCO_3$, 5.9 mM KCl, 4 mM NaH_2PO_4, 1 mM Na_2HPO_4 and 10 mM glucose. After a 30 min equilibration period with high Pi buffer, ^{31}P NMR spectra were collected for 45 min and then the buffer was switched to control buffer again. Following a 30 min. equilibration period with control buffer, ^{31}P NMR spectra were collected for the next 30 min.

The concentrations of extracellular free calcium (free $[Ca^{2+}]o$) and extracellular free magnesium (free $[Mg^{2+}]o$) were calculated according to known acid dissociation constants and binding constants (11). The ratio of NaH_2PO_4/Na_2HPO_4 was determined to yield a pH of 7.4 with concentrations of extracellular Pi ([Pi]o) of 1.2 and 5.0 mM, respectively (11), respectively, in both control and high Pi buffers free $[Ca^{2+}]o$, free $[Mg^{2+}]o$ and pH were adjusted to the level of 1.07 mM, 0.5 mM and 7.4, respectively, as described before. (11).

NMR spectroscopy

^{31}P NMR spectra were obtained under a 2-tesla field using a Photal BEM 250/80 spectrometer (Ohtsuka electronics CO. LTD, Osaka, Japan) operating in the pulsed Fourier transform mode. With this field, phosphorus nuclei resonate at a frequency of approximately 34.53 MHz. Spectra were collected using a pulse repetition of 3.0 s corresponding to a 45° pulse angle and 300 scans over a total acquisition time of 300 × 3.0 s (15 min) for each experimental condition. The creatine phosphate (PCr) signal was used as the internal standard of chemical shift.

Analyzing ^{31}P NMR spectra, the LPZAR method which was proposed by Tang and Norris (17) was used. The basic concept of the LPZAR method is that unmeasured parts of free induction decay (FID) signals are predicted and a signal with a finite number of dumped sinusoids can be approximated and extended to an infinite sequence. As the result,

the spectra obtained by the LPZAR method exhibit sharper peaks with less noise than those obtained by the conventional FFT method. The chemical shift, and the maximum and the full width at the half of the peak were estimated by the least squares method based on the modified Gauss-Newton method (18).

Estimation of intracellular free [Mg^{2+}]

Free [Mg^{2+}]i was determined from the Mg^{2+} dependent chemical shift of the β peak relative to the α peak of ATP using the following equation (12,19).

$$[Mg^{2+}] = \frac{\delta ATP - \delta \alpha\beta}{\delta \alpha\beta - \delta Mg^{2+}ATP} Kd \quad (1)$$

where Kd is the dissociation constant of Mg^{2+}ATP. σ ATP is the chemical shift of the β-phosphate relative to the α-phosphate of ATP in the absence of Mg^{2+}. δ Mg^{2+}ATP is the chemical shift of the β-phosphate relative to the α-phosphate in ATP which is fully combined with Mg^{2+}, and δ αβ is the observed chemical shift between α-and β-phosphate of ATP. The values for δ ATP and δ Mg^{2+}ATP were 10.8148 ppm and 8.3210 ppm, respectively (13,14). For Kd of Mg^{2+}ATP we used the value of 45 μM which was determined by Gupta et al. (20).

Values were presented as the mean ± SEM and were analyzed by ANOVA for comparisons between control perfusion and high Mg^{2+} or high Pi perfusion. A p-value less than 0.05 was considered statistically significant.

RESULTS

Typical ^{31}P NMR spectra obtained from FFT and LPZAR in control perfusion of isolated rat heart are shown in Figure 1. The major peak assignments are given in the figure. Spectral peak resonance was measured relative to that of PCr in parts per million (ppm). ^{31}P NMR spectrum calculated by LPZAR had less noise, less fluctuation of baseline, sharper peaks, much higher resolution as compared with FFT. Figure 2 shows the effects of [Mg^{2+}]o on NMR spectra. To distinguish changes produced in the α - and β -peaks of ATP more clearly, spectra were shown in an expanded scale. High Mg^{2+} perfusion produced a leftward shift in the β-peak of ATP, and changed δ αβ from 8.5265 ppm to 8.4643 ppm, which resulted in calculated free [Mg^{2+}]i from 0.54 mM to 0.73 mM, respectively. The mean value of free [Mg^{2+}]i in 4 rat hearts was 0.51 ± 0.04 mM during control perfusion and significantly increased to 0.66 ± 0.05 mM with high Mg^{2+} perfusion ($p<0.05$). The ratio of Mg^{2+}ATP concentration to total ATP concentration ([Mg^{2+}ATP]/[ΣATP]) was calculated to be 91.7 ± 0.5 % during control perfusion and 93.2 ± 0.5 % with high Mg^{2+} perfusion when [Mg^{2+}]o was increased to 20 mM.

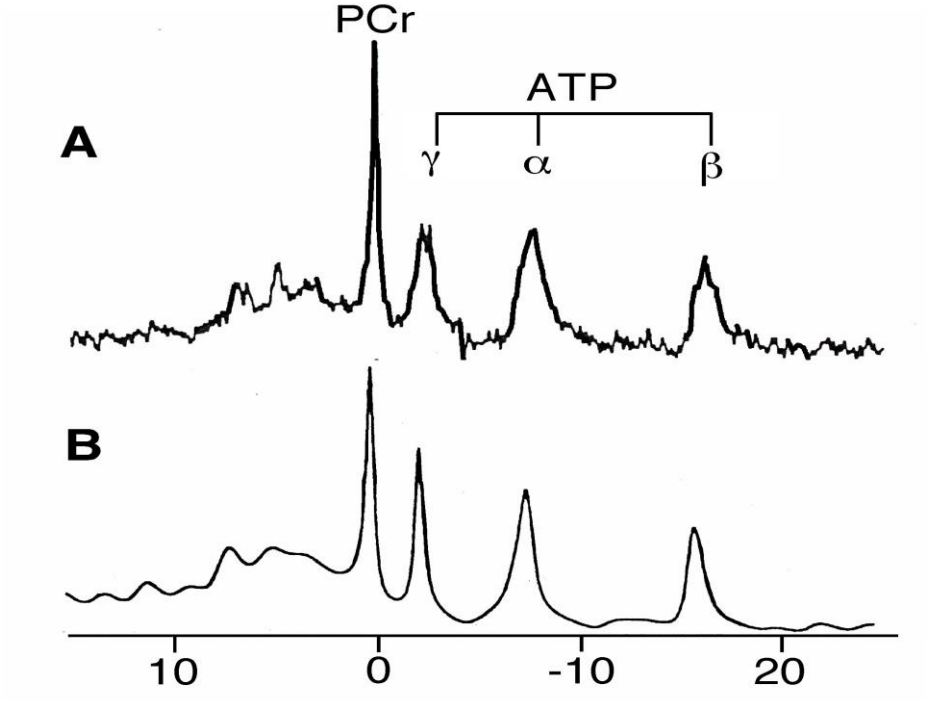

Figure 1. Comparison of ^{31}P NMR spectra calculated with fast Fourier transform (FFT) method (A) and with the linear prediction z-transform (LPZAR) method (B).

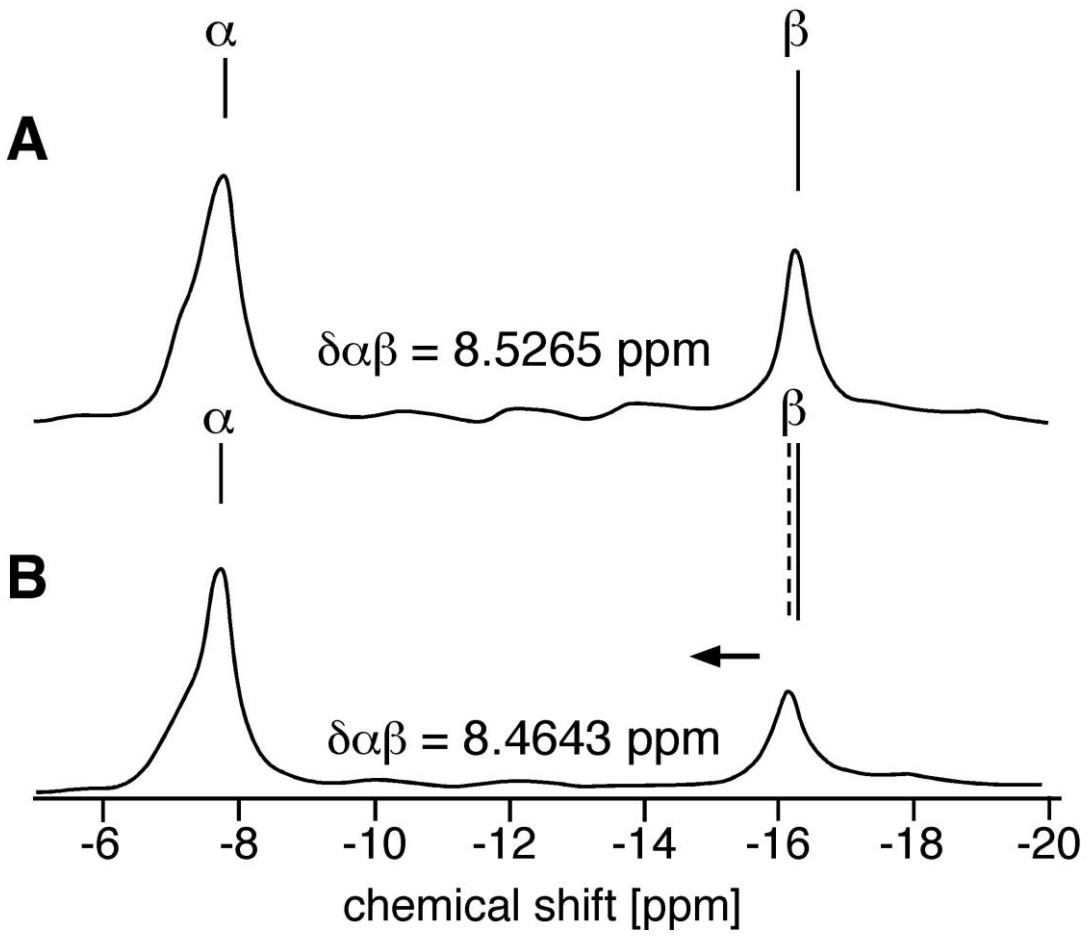

Figure 2. Effect of excess Mg^{2+} on NMR spectra obtained from LPZAR method in isolated rat heart. The α- and β-peaks of ATP are shown on an expanded scales. A: $[Mg^{2+}]o$ = 0.52 mM, B: [Mg^{2+}]o = 20 mM. Vertical solid lines indicate the α- or β-peak of ATP and broken line in B indicates the β-peak during high Mg^{2+} perfusion. High Mg^{2+} Perfusion produced a leftward shift in the β-peak of ATP.

Figure 3 demonstrates consecutive spectra obtained from LPZAR in control perfusion (Figure 3A), high Pi perfusion (Figure 3B) and subsequent control perfusion (Figure 3C). The α-and β-peaks of ATP were shown on an expanded scale. High Pi perfusion produced a rightward shift in the β-peak of ATP (Figure 3B) and the subsequent control perfusion shifted the β-peak back to the left (Figure 3C). δ αβ changed from 8.4910 ppm to 8.5345 ppm and returned to 8.4870 ppm.

Figure 4 summarizes the changes of free $[Mg^{2+}]i$, intracellular total ATP (ΣATP) and $[Mg^{2+}ATP]/[\Sigma ATP]$ in control perfusion, high Pi perfusion and subsequent control perfusion in 6 rat hearts.

Calculated free $[Mg^{2+}]i$ decreased significantly from 0.53 ± 0.05 mM in control perfusion to 0.41 ± 0.03 mM in high Pi perfusion ($p<0.05$) and returned to 0.60 ± 0.05 mM in the subsequent control perfusion. While intracellular ΣATP in initial perfusion was 100 %, the relative values of intracellular ΣATP in high Pi perfusion and the subsequent control perfusion to that in initial perfusion were 104.9 ± 5.6 % and 94.2 ± 7.3 %, respectively. There were no significant changes in intracellular = ATP during various perfusions. $[Mg^{2+}ATP]/[\Sigma ATP]$ also decreased significantly from 91.7 ± 0.7 % in control perfusion to 89.8 ± 0.7 % in high Pi perfusion ($p<0.05$) and returned to 92.8 ± 0.6 % in the subsequent control perfusion.

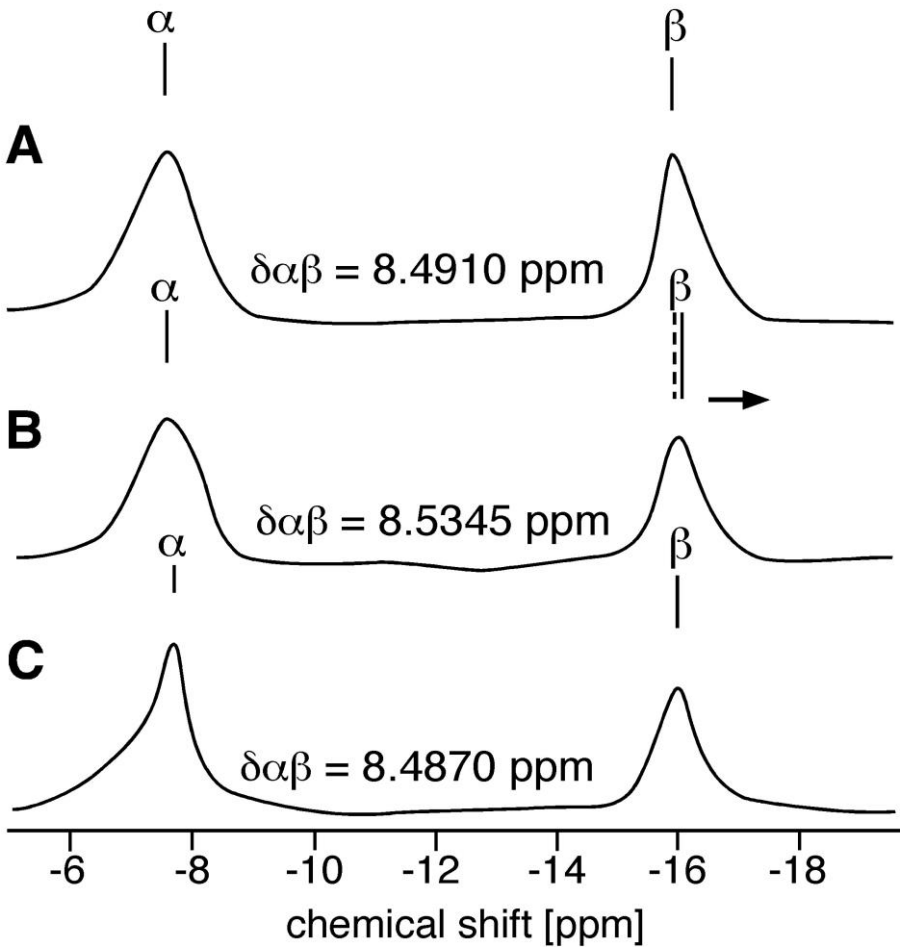

Figure 3. Effect of excess Pi on NMR spectra obtained from LPZAR method in isolated rat heart. The α- and β-peaks of ATP are shown on an expanded scales. A: [Pi]o = 1.2 mM, B: [Pi]o = 5.0 mM and C: [Pi]o = 1.2 mM. Vertical solid lines and a broken line indicate the same with Fig. 2. Pi of perfusate was increased from 1.2 mM (A) to 5.0 mM (B) and then returned to 1.2 mM (C). High Pi perfusion produced a rightward shift in the β-peak of ATP. When [Pi]o was returned to 1.2 mM, the β-peak was shifted back to the left.

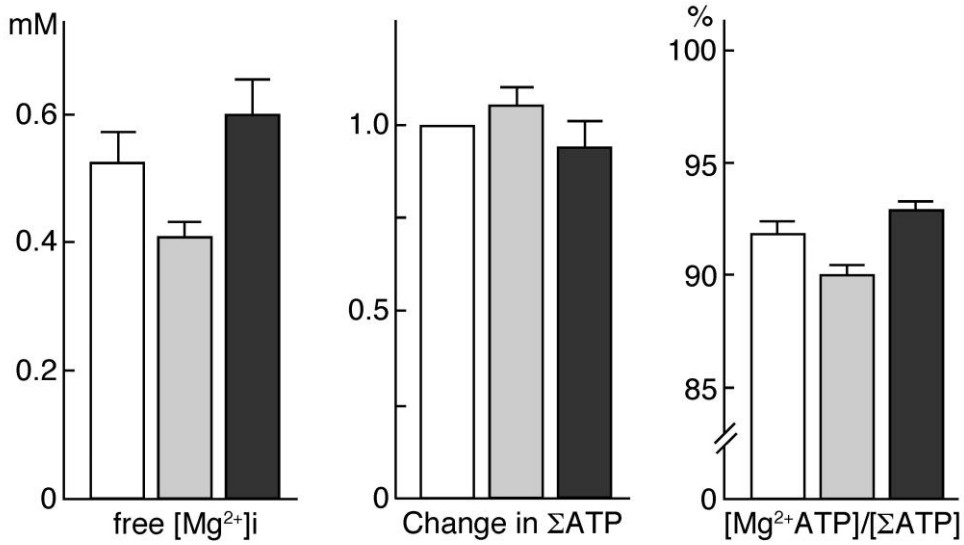

Figure 4. The effect of [Pi] in free [Mg^{2+}]i, intracellular total ATP (ΣATP) and [Mg^{2+}ATP]/[ΣATP] Six hearts were perfused with control, high Pi and control buffer in sequence. Each data was presented as the mean ± SEM, when Pi of perfusate increased from 1.2 mM (□) to 5.0 mM (▨) and then returned to 1.2 mM (■). ATP indicates the relative value of intracellular total ATP (ΣATP) in each perfusion, while intracellular ΣATP in initial perfusion was 100 %. [Mg^{2+}ATP]/[ΣATP] means the ratio of Mg^{2+}ATP concentration to total ATP concentration and is expressed as %.

DISCUS SION

^{31}P NMR spectroscopy is capable of determining the number of phosphorus nuclei and has been used to estimate intracellular free Mg^{2+}, pH, and steady-state unidirectional reaction rates of a number of key enzymes of metabolism *in vivo* (12-14, 20-22). FFT method is commonly used to convert sampled time-domain signals into frequency domain data in NMR spectroscopy. However, identification of the peak in some spectra of ^{31}P NMR is difficult, particularly in spectra with low signal-to-noise ratios and baseline shifts. Furthermore, when time-domain signals decrease quickly or are truncated, the analysis of ^{31}P NMR spectra using FFT method has limitation since phosphorus is a low sensitivity nucleus (23).

Thus, we applied LPZAR method to analyze peaks. The basic concept of LPZAR method is that a signal with a finite number of dumped sinusoids is approximated by a linear combination of previous data points.

The LPZAR requires only the initial parts of the FID signals which contain important signals and less noise than the other parts, in contrast to the FFT method which uses entire FID signals containing noisy signals in the tail parts (11). Clear peaks of PCr, γ-, α- and β-ATP from the beating perfused rat heart were visible by using LPZAR. The use of ^{31}P NMR is attractive in the estimation of free $[Mg^{2+}]i$, since it is non-destructive to the heart and can be repeated in time (12,19,24,25). Reliable value for δαβ in the analysis of ^{31}P NMR spectra was able to obtain by the LPZAR method. The δαβ is little affected by small pH changes, because the α- and β-phosphorus resonances of ATP undergo only slight and similar shifts with changes in pH near 7.4 (26). Measurement of free $[Mg^{2+}]i$ by δαβ was justified in various pathological conditions if δATP, δ Mg^{2+}ATP and Kd are constant (16).

Values of free $[Mg^{2+}]i$ obtained during initial perfusion in two groups (0.51 ± 0.04 mM and 0.53 ± 0.05 mM) were well in agreement with values of 0.5 to 0.6 mM which were obtained by others using ^{31}P NMR in isolated hearts (27-29). Our previous study suggested the presence of the Mg^{2+}-Pi exchange mechanism across the plasma membrane in heart muscle (9) In that study free $[Mg^{2+}]i$ was biochemically estimated from intracellular citrate/isocitrate ratio. The present study based on direct measurement of free $[Mg^{2+}]i$ with the NMR method should free $[Mg^{2+}]i$ decreases when [Pi]o increases; supporting the idea of the existence of the Mg^{2+}-Pi exchanger in the plasma membrane.

In conclusion, the LPZAR method provided more correct information for an analysis of ^{31}P NMR spectra than the FFT method under technically difficult conditions such as short acquisition time and low magnetic field. Using the LPZAR method, we were able to detect γ-,α- and β-peaks of ATP and to estimate free $[Mg^{2+}]i$ in perfused rat heart. The decrease of free $[Mg^{2+}]i$ found here during 5.0 mM Pi perfusion may support the existence of Mg^{2+}-Pi exchanger on the ion movement across the plasma membrane of rat heart.

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