PHOSPHORUS NMR STUDIES ON PERFUSED HEART

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Received December 21,1976

SUMMARY

Phosphorus nuclear magnetic resonance measurements at 129MHz have been made on small beating rat hearts, perfused by the Langendorff technique. Good spectra, giving the levels of ATP, creatine phosphate and inorganic phosphate can be collected in as little as 10-20min and the heart can be maintained in a steady state in the spectrometer for at least 5 hours. In a good preparation, the ratios of the β-phosphate of ATP, to creatine phosphate and inorganic phosphate are 1:1.8:1.8 which compare well with the data obtained by analyzing a freeze-clamped extract by NMR. The recovery of metabolites, after the induction of global ischaemia, has been followed.

We have reported that nuclear magnetic resonance can be used to observe phosphorus-containing metabolites in skeletal muscle (1). Since then the wide applicability of the method to a variety of intact tissue and subcellular preparations has been demonstrated (2-5). More recently we have examined the feasibility of carrying out ^{31}P NMR measurements on rat heart preparations and compared the properties of normoxic and anoxic cardiac tissue (6). Use of NMR to observe changes in phosphate levels of skeletal muscle following contraction (7,8) has led naturally to application of the NMR method to studying some of the metabolites in perfused rat heart preparations. The purpose of this communication is to report rapid collection of ^{31}P NMR data from small, beating rat hearts. Our results demonstrate the feasibility of detailed investigations on cardiac metabolism using the NMR technique.

MATERIALS AND METHODS

Male Wistar rats weighing about 30g were anaesthetized with diethyl ether and then injected with heparin (1 unit/g.body weight) via the left

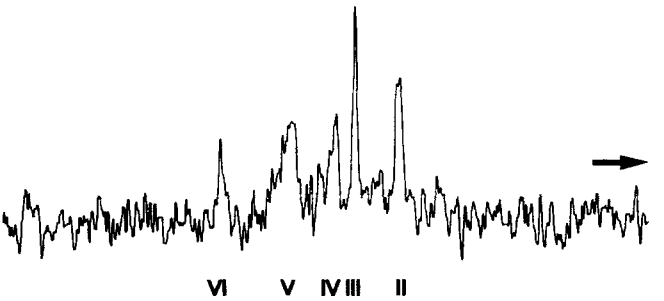

Fig. 1

129 MHz phosphorus NMR spectrum of 160mg rat heart perfused by the Langendorff technique at 37°C. Sweep width 10 kHz. 1000 60° radio-frequency pulses were applied at 4s intervals.
Peak assignments: II = inorganic phosphate, III = creatine phosphate, IV = γ-ATP, V = α-ATP, VI = β-ATP.
(Arrows in spectra indicate the direction of increasing frequency).

femoral vein. The hearts were removed and placed in ice-cold perfusion medium until contraction had ceased. They were then mounted on a glass cannula (inner diameter 0.7mm and outer diameter 1.0mm) and perfused in the retrograde Langendorff mode (9) at 37°C, with Krebs-Henseleit buffer containing 11mM glucose and gassed with 95% O_2:5% CO_2. The buffer was made up using Umbreit's method (10) to prevent precipitation of calcium salts and was filtered through a 5μm Millipore filter. The pressure head was 85cm of water and the coronary effluent was not recirculated. Hearts began to beat spontaneously within 30s of supply of warm buffer. After a 5minute equilibration period the hearts were immersed in buffer in an NMR tube (inner diameter 8mm) modified to allow the coronary flow to drain out while still maintaining the heart under fluid. The tube was then placed in the NMR spectrometer and cardiac function assessed by measurements of coronary flow rate and heart rate. Coronary flow remained constant at 20ml/min/g.wet weight of heart over periods up to 5 hours, and heart rate was also constant during this time at 200 beats/min. Inspection of the heart after 5 hours' perfusion revealed no dark ischaemic patches.

Hearts used for perchloric acid extraction were from 300g rats and were perfused non-immersed for 5 minutes by the method described above before freeze clamping with Wollenberger tongs (11). The cannulae used here were larger (inner diameter 1.0mm., outer diameter 1.8mm.) Frozen tissue was ground in a percussion mortar and homogenized in 3 volumes of 3% perchloric acid. The solids were then spun down and the supernatant neutralized to pH 7 with potassium hydroxide, and concentrated by freeze-drying.

NMR spectra of perfused hearts were recorded at 129MHz on a spectrometer constructed in Oxford (12). The spectrometer was operated in the Fourier Transform mode and was interfaced with a Nicolet B-NC 12 computer. Spectra were collected without the use of a field-frequency lock. (Field drift was 10 Hz/hour maximum). Phosphorus NMR spectra of extracts were recorded at 36.4 MHz on a Bruker WH-90 spectrometer using a 9mm inner diameter sample tube and external deuterium oxide field-frequency lock. The spectra of figures 1-3 are all proton-coupled.

RESULTS AND DISCUSSION

Fig.1 shows the ^{31}P NMR spectrum of a 160mg rat heart perfused at 37°C. The peaks in the spectrum can be assigned to ATP, creatine phosphate and inorganic phosphate (1). The frequency of the inorganic phosphate signal is sensitive to the state of ionization of the molecule and shows that the intracellular pH in the perfused heart is 7.4. As in skeletal muscle (1), the three ATP resonances coincide with those of MgATP showing that almost all ATP is complexed to a divalent cation.

Attempts have not yet been made to quantitate absolute phosphate concentrations. Values for relative signal intensities are, however, given in Table 1. Precision of these ratios requires the absence of artificial reduction of signal intensities due to saturation effects, i.e. markedly different spin-lattice relaxation rates for the signals compared. Partial saturation of some signals does occur when spectra are collected with 2s intervals between pulses. Accurate concentration ratios are given by integration of '4s spectra'(Table 1).

The concentration ratios for perfused heart are in broad agreement with those for perchloric acid extracts (Fig.2). The β-ATP signal is taken as the concentration standard for ATP since the α- and γ - signals are overlapped by those of ADP,and the α-signal also coincides in frequency with signals of pyrophosphate diesters such as NAD and NADH. The high value of [creatine phosphate]/[ATP] ($\simeq$ 1.8) and low [inorganic phosphate]/[ATP] ($\simeq$ 1.8) are indices of efficient cardiac respiration. These values remained constant throughout the perfusion period and are therefore - along with heart and coronary flow rates - a further indication of a functional steady state of the heart. The values are also similar to those obtained from biochemical determinations on freeze-clamped extracts (13). A marked discrepancy occurs for '[α-ATP]'/[β-ATP] ratios: values are consistently higher for hearts from young (30g) animals. This age dependence effect in '[α-ATP]'/[β-ATP] has also been observed in rat skeletal muscle (unpublished observations) and is accounted for by additional

TABLE 1

Integrals of phosphorus signals relative to β-ATP

160mg rat hearts direct observation	β-ATP	α-ATP	γ-ATP	Creatine phosphate	Inorganic phosphate	Sugar phosphate and nucleotide monophosphate
4s pulse intervals	1	3.6±0.5	1.7±0.3	1.9±0.3	1.8±0.3 *	n.o.
2s pulse intervals	1	1.6±0.3	1.1±0.2	1.4±0.3	1.6±0.3 *	n.o.
800mg rat hearts 3% perchloric acid extraction 4s pulse intervals	1	1.5±0.2	1.2±0.2	1.7±0.3	2.2±0.3	0.9±0.2

n.o. = not observable

* Corrected for inorganic phosphate in Krebs-Henseleit buffer.

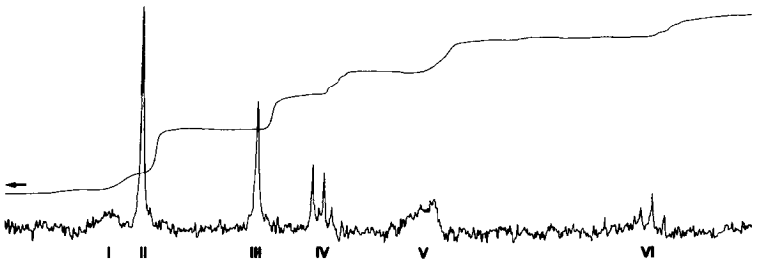

Fig. 2

36.4MHz phosphorus NMR spectrum of a perchloric acid extract of five 800mg rat hearts (20mM HEPES, 100mM EDTA, pH 7.6). Sweep width 1.25 kHz. 4000 70° radio-frequency pulses applied at 4s intervals.
Peak assignments: I = sugar phosphate and nucleotide monophosphates, II = inorganic phosphate, III = creatine phosphate, IV = γ-ATP and β-ADP, V = α-ATP and α-ADP, VI = β-ATP.
The upper line is a running integral of the spectrum.

signals overlapping that of α-ATP, at least partly NAD/NADH.'[γ-ATP]/[β-ATP] ratios are also greater than unity. At least part of the extra γ-signal may be β- ADP and resolved signals from this nucleotide phosphate are visible in Fig. 2. The nucleotide signals of perfused heart spectra are substantially those from unbound molecules. A detailed estimate of phosphate concentrations and compartmentation requires absolute signal intensities of tissue spectra.

Fig. 3 shows phosphorus spectra of hearts subjected to periods of global ischaemia with and without return of flow. Fig. 3a represents a heart during the first half hour of recovery from 15 minutes ischaemia. It shows raised levels of sugar phosphates due to activation of glycogenolysis during the ischaemic period, but recovery, represented by creatine phosphate and ATP levels, is however substantially complete. Fig. 3b represents a heart after 20 minutes ischaemia with no reflow. Only inorganic phosphate, sugar phosphate and nucleotide monophosphate signals are detectable. Fig. 3c is a broad line sweep of an ischaemic heart spectrum. The broad component ($\Delta\nu_{\frac{1}{2}}$ = 2.5 kHz) is the signal underlying the free phosphate resonances in 10 kHz spectra. The breadth of this signal is consistent with its assignment to the relatively slow-tumbling phosphates of phospholipids and nucleic acids.

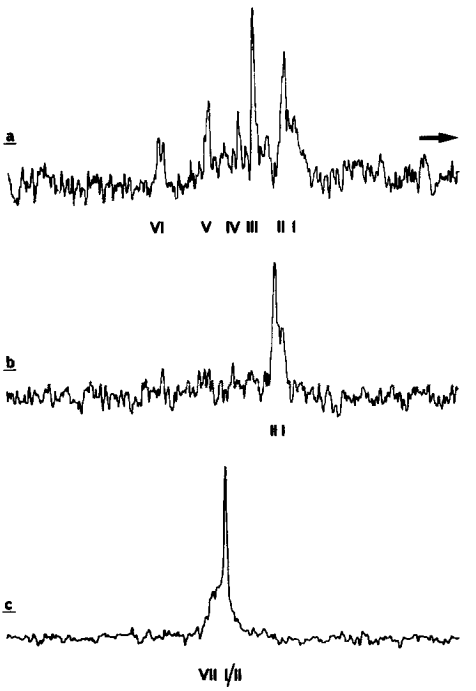

Fig. 3

129 MHz phosphorus NMR spectra of a 160mg rat heart under different ischaemic conditions.

a. 15 minutes ischaemia plus reflow
(1200 60° pulses at 2s intervals. 10 kHz sweep).

b. 20 minutes ischaemia without reflow
(600 60° pulses at 2s intervals. 10 kHz sweep).

c. Wide sweep spectrum collected after spectrum b
(6000 30° pulses at 0.5s intervals. 50kHz sweep).

Peaks I and II are merged on the wide-sweep scale.
VII is the down-frequency shoulder of a broad signal from slow-tumbling phosphates.

Our results demonstrate that ^{31}P NMR can be used to follow the metabolic state of perfused heart tissue. The main advantage of the method is that the time course of events associated with ischaemia and recovery can be followed on a single heart at physiological temperature.

ACKNOWLEDGEMENTS

This research was supported in part by the Science Research Council. P.J.S. is an ICI Research Fellow. We thank Mrs. E.E. Richards for the use of the Bruker WH-90 instrument. Dr. D.I. Hoult gave invaluable help and advice

in the use of the 129 MHz instrument. We also thank Dr. R.E. Richards for his interest in the project. Dr. W.E. Jacobus kindly communicated his results to us in advance of publication. Our observations are in broad agreement with his, but there are several experimental differences which make detailed comparisons inappropriate at this time.

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