^{31}P NMR SATURATION TRANSFER MEASUREMENTS OF THE STEADY STATE RATES OF CREATINE KINASE AND ATP SYNTHETASE IN THE RAT BRAIN

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1. Introduction

There are two theories concerning the function of phosphocreatine (PCr) in cellular energy metabolism. In one PCr is seen as a reservoir, functioning to maintain constant cytoplasmic ATP concentrations. The alternate theory postulates that PCr acts as an energy shuttle between the site of ATP production in the mitochondria and the site of utilization in the cytoplasm (reviews [1,2]). Both theories require that the exchange flux in the creatine kinase (CPK) reaction be considerably greater than the rate of ATP hydrolysis. This is necessary in order to stabilize ATP concentrations during transitions in workload, or during periods when ATP synthesis is impaired, such as ischaemia. Calculations of cytoplasmic phosphorylation potential [3] and measurements of intracellular pH [4] have been based on the assumption that the CPK reaction is near equilibrium. It is now possible to test this assumption in vivo using the technique of ^{31}P NMR saturation transfer; the flux through the CPK reaction was 5 times the steady state rate of ATP turnover in the Langendorff perfused rat heart [5].

The total extractable activities of CPK in the rat brain and heart are similar; 500 μmol . g^{-1} . min^{-1} in heart [6] and 200 μmol . g^{-1} . min^{-1} in brain [7], measured at 25°C. The mitochondrial form of the enzyme accounts for 30–40% of the total activity in the heart [1] and 5% in brain [7]. However, mitochondrial CPK activity in both organs is very similar on a per gram mitochondrial protein basis [8]. Despite these similarities, the metabolic response to a decrease in the efficiency of cellular energy transduction, brought about by ischaemic intervention, is quite different in the two organs. In the ischaemic heart the PCr concentration decreases before any change is observed in ATP [9,10], whereas in the ischaemic brain PCr and ATP fall in concert [11,12]. A number of factors could account for this observation, including differences in tissue heterogeneity between the two organs, or a relatively slower flux through CPK in the brain. We have investigated the latter possibility by measuring the fluxes through the CPK and ATP synthetase reactions in the rat brain in vivo. We have also compared our measurements of ATP synthetase to measurements of cerebral oxygen consumption from the literature.

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2. Methods

2.1. *Animals*

Adult male Wistar rats (300–400 g body wt) were used. The animals were allowed free access to food and water. Anaesthesia was induced with sodium pentobarbitone (35–40 mg/kg) and the animals were then mounted vertically in a Perspex cradle, care being taken not to restrict ventilatory movements. The head was fixed in position with the aid of 2 nylon screws which acted as supports at the base of the skull. A surface coil [13] was positioned over the brain. Anaesthesia was maintained throughout the experiment with 0.5% halothane carried in 50% N_2O/50% O_2, delivered through a nosecone. Body temperature was maintained by a flow of warm air over the animal.

2.2. *Nuclear magnetic resonance*

^{31}P NMR spectra were accumulated on a spectrometer constructed in Oxford [14], utilizing a wide-bore,

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superconducting magnet (4.3 T), operating at 73.84 MHz in the Fourier transform mode. A two-turn, 15 mm diam. surface coil [13] tuned to the ^{31}P frequency was used both as transmitter and receiver. The coil was designed so that only signals from the brain were observed. Field homogeneity was optimized by observing the water proton signal in the tissue [15].

The theory of saturation transfer has been described [16,17]. Pseudo-first order rate constants were derived using the relationships:

$$M^+/M^\circ = T_{1M}/T_1 \text{ and } 1/T_{1M} = 1/T_1 + k$$

where:

M^+/M° represents the fractional magnetization after saturation transfer;

T_1, the intrinsic spin-lattice relaxation time (in the absence of chemical exchange);

T_{1M}, the measured spin-lattice relaxation time in the presence of appropriate saturating radiation at the exchanging site,

k, the pseudo-first order rate constant

Fluxes were determined as the product of the rate constant and the estimated intracellular concentration of the appropriate metabolite. Radiofrequency radiation, noise modulated to 20–40 Hz bandwidth, of sufficient strength to completely saturate the irradiated resonance was applied continuously except during data acquisition. Excitation pulses of nominal 90° flip angles were repeated at intervals of ~5 T_1. Transfer of saturation was measured by comparing the area of the resonance exchanging with the saturated site to that obtained with control irradiation, the two irradiating frequencies being symmetrically placed about the resonance. This procedure compensates for non-specific, direct saturation effects. Spectra were accumulated in blocks of 200 scans (400 scans total) for estimation of the extent of saturation in the CPK reaction. Blocks of 100 scans (600 scans total) were used to measure saturation transfer in the ATP synthetase reaction. T_1-Values were measured using the saturation recovery method [18]; a homospoil pulse applied to the shim coils was used to destroy residual xy magnetization. To compensate for the inherently poor B_1 homogeneity of the surface coil [13], a composite 90° pulse [19] was applied just prior to the homospoil pulse. An iterative three-parameter-fit equation [20] was used for T_1 calculations. These procedures are essential for accurate determinations of T_1-values with surface coils (R.W.B. et al., unpublished).

Experiments were generally run for <6 h except for the measurement of the T_1 for inorganic phosphate which took 8 h. Thus it was not possible to make all measurements on any individual animal. The data are presented as means ± SD.

3. Results

The ^{31}P NMR spectrum of the rat brain contains signals from PCr, the three phosphate groups of ATP, inorganic phosphate (P_i), sugar phosphates, NAD^+ + NADH and an unidentified resonance of variable intensity in the phosphodiester region (fig.1). Saturation of PCr reduces the ATP γ-phosphate intensity to 76.1% of its control value (fig.1, table 1). The transfer of saturation is most clearly seen in the difference

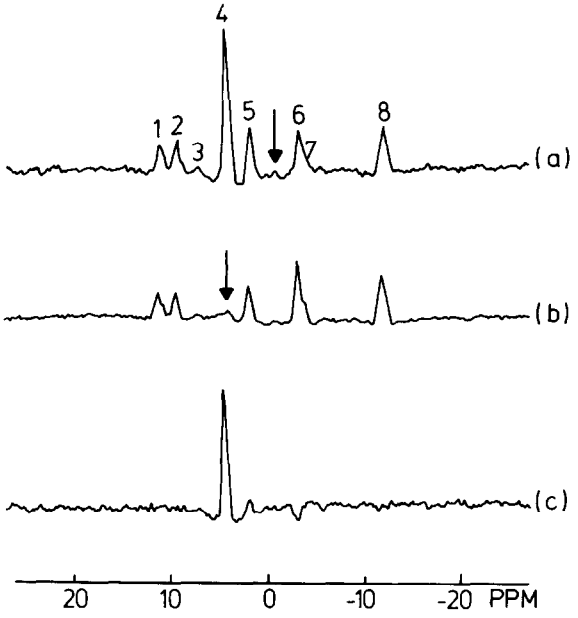

Fig.1. ^{31}P NMR spectra of the in vivo rat brain showing the transfer of saturation to the γ-phosphate of ATP on irradiation of PCr: (a) control spectrum, irradiation at arrow; (b) saturating irradiation applied to PCr; (c) difference spectrum, (a–b), showing saturation transfer. The convolution difference technique was used to eliminate the broad components arising from the skull in (a) and (b). Resonances: (1) sugar phosphates; (2) P_i; (3) phosphodiester; (4) PCr; (5) ATP γ-phosphate; (6) ATP α-phosphate; (7) NAD^+ + NADH; (8) ATP β-phosphate. The spectra are the result of 400 data accumulations.

Table 1
Results of the saturation transfer experiment

	M^*/M^0	T_{1M} (s)	T_1 (s)
ATP	0.761 ± 0.038 (n = 8)	1.05 ± 0.11 (n = 7)	1.4
PCR	0.436 ± 0.056 (n = 7)	2.19 ± 0.24 (n = 6)	5.0
P_i	0.646 ± 0.034 (n = 7)	1.92 ± 0.11 (n = 5)	3.0

M^*/M^0, the residual magnetization expressed as a fraction of control intensity; T_{1M}, the measured spin-lattice relaxation time in the presence of saturating irradiation at the exchanging site; T_1 the calculated intrinsic spin lattice relaxation time. Values as means ± SD

spectrum (fig.1c). The small inverted ATP α-phosphate resonance in the difference spectrum presumably results from the direct effects of the control irradiation.

When the ATP γ-phosphate resonance is saturated PCr and P_i are reduced to 43.6% and 64.6% of their control values, respectively (fig.2, table 1). We consistently observed transfer of saturation to both the α- and β-phosphates of ATP on irradiation of the ATP γ-phosphate (fig.2c). The transfer to the ATP β-phosphate may result from adenylate kinase, creatine kinase or ATP synthetase because ADP β-phosphate, which we cannot see in the spectrum, is saturated together with ATP γ-phosphate. It is, however, more difficult to explain transfer to ATP α-phosphate on the basis of chemical exchange. This suggests that these apparent saturation effects may be the result of a negative ^{31}P nuclear Overhauser enhancement.

Spin-lattice relaxation times (T_{1M}) were measured for PCr, P_i and ATP γ-phosphate in the presence of saturating irradiation at the exchanging site. These

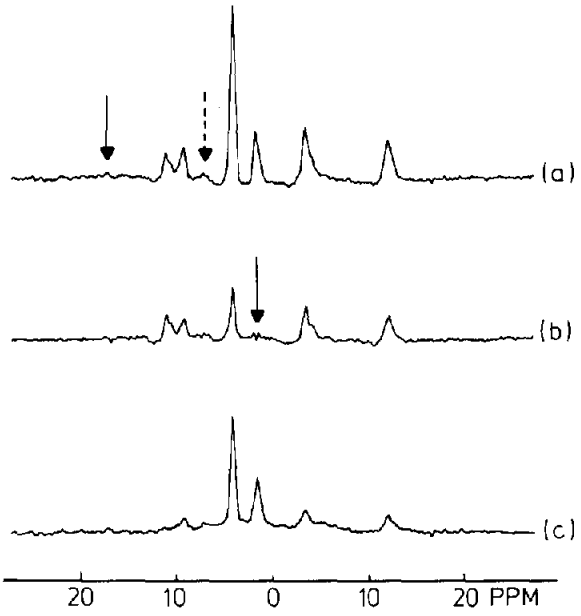

Fig.2. ^{31}P NMR spectra of the in vivo rat brain showing transfer of saturation on irradiation of ATP γ-phosphate. (a) Control for direct saturation effects on P_i, irradiation at solid arrow. (The control for direct saturation effects on PCr is not shown in the figure. Irradiation was applied at the dashed arrow.) (b) Saturating irradiation applied to ATP γ-phosphate; (c) difference spectrum, (a–b) showing saturation transfer. The spectra are the result of 600 data accumulations.

results, together with the calculated intrinsic T_1-values, are given in table 1. From these data we have calculated the unidirectional, pseudo-first order rate constants for the CPK and ATP synthetase reactions (table 2). In order to estimate the unidirectional fluxes through these reactions we have made one assumption; that the NMR ATP signal is equivalent to the ATP concentration obtained in funnel-frozen

Table 2
Calculated unidirectional rate constants *(k)* and fluxes *(F)* for the creatine kinase (CPK) and ATP synthetase reactions

	CPK (forward)	CPK (reverse)	ATP synthetase
k (s^{-1})	0.26	0.23	0.18
F (μmol . g wet wt^{-1} . s^{-1})	1.64	0.68	0.33

The flux calculations assume an in vivo [ATP] of 3 μmol/g wet wt. The molar ratios P_i/ATP and PCr/ATP are given in the text. The forward direction of the CPK reaction is the direction of PCr hydrolysis

or freeze-blown brain extracts. When due precautions are taken, this seems the least variable metabolite, and there are many studies indicating that the concentration of ATP in the rat cerebral cortex is ~3 μmol/g wet wt [4,21]. Spectra were collected under fully relaxed conditions to determine the concentrations of PCr and P_i relative to ATP. The following molar ratios were obtained: PCr/ATP = 2.12 + 0.28 and P_i/ATP = 0.595 + 0.055 (n = 10). Based on these results, the flux estimates show that the forward flux through CPK is 5 times the steady state rate of ATP synthesis (table 2). Note that this relationship is not sensitive to the assumed concentration of ATP.

4. Discussion

The relative unidirectional fluxes through the CPK and ATP synthetase reactions in the rat brain are nearly identical to those reported for the perfused rat heart [5], and support the contention that the CPK reaction is near equilibrium in the cell. Thus the differences in the pattern of metabolic events during ischaemia in the heart and brain are not the result of a relatively lower CPK activity in the brain. A likely reason for the apparent parallel decrease of PCr and ATP is tissue heterogeneity with respect to energy demands in the brain. In the partially ischaemic brain unequal perfusion of the so called watershed areas is well known [22].

The discrepancy between the forward and reverse flux through CPK in the steady state has been observed in the heart before [23–25]. It may arise from cellular compartmentation of ATP pools [23], one of which is not accessible to the enzyme. Alternatively, while the only known reaction involving PCr is CPK, ATP is involved in many reactions, e.g., ATP hydrolysis, adenylate kinase and nucleoside diphosphate kinase. All of these reactions must act as additional relaxation processes for ATP magnetization, resulting in a spuriously low value for the measured reverse CPK flux. We are not yet able to distinguish between these two alternative explanations.

The ATP synthetase flux which we have measured represents the total unidirectional rate at which P_i is incorporated into ATP. If one makes the following assumptions: glucose is the sole substrate for brain, metabolism is entirely aerobic, the ADP:O ratio is 3 and 32 of 36 ATP molecules generated in the complete combustion of glucose are derived from oxidative phosphorylation, then the ATP synthetase flux corresponds to a cerebral O_2 consumption of 2.9 μmol $O_2 \cdot g^{-1} \cdot min^{-1}$. This is close to the rates of 4.6 μmol $O_2 \cdot g^{-1} \cdot min^{-1}$ for rat cerebral cortex [26] and 3.4 μmol $O_2 \cdot g^{-1} \cdot min^{-1}$ for the whole brain [27] measured by conventional washout techniques. Two factors complicate direct comparison of these results. Because of the placement of the *rf* coil and the characteristics of the B_1 field of the surface coil [13] most, but not all, of the NMR signal we observe will originate from the cerebral cortex. Our experiments were carried out under light halothane anaesthesia, those in [26,27] under 70% N_2O/30% O_2. The latter has no measureable effect on cerebral oxygen consumption in rats [28], while halothane produces significant, dose-dependent reductions in cerebral oxygen consumption [29]. Halothane at 0.6% was found to reduce cerebral oxygen consumption in rats by 25% [29]. This is the maximum reduction we would expect in our experiments. Taking this into account, our result agrees well with conventional measurements which would predict O_2 consumption between 2.6–3.5 μmol $\cdot g^{-1} \cdot min^{-1}$.

What are the potential sources of error in the ATP synthetase measurement? The 2-phosphate of 2,3-diphosphoglycerate (2,3-DPG) has a chemical shift in the same region as P_i [30] and it has been suggested that it contributes to signal in this region [13]. The concentration of 2,3-DPG in the erythrocytes of the rat is ~7 mM [31] and the brain blood volume is ~3% [32]. Assuming a haematocrit of 40% and a uniform distribution of blood throughout the brain, the concentration of 2,3-DPG which we would detect by NMR would be <5% of the mean estimated P_i concentration. This is well below the error of measurement of P_i which we estimate to be ± 10%. ATP is not the only nucleotide triphosphate (NTP) present in the brain. GTP, UTP and CTP together comprise ~20% of the total pool [21]. These compounds are indistinguishable from ATP within the limits of our NMR resolution. Thus we measure unidirectional exchange between the whole NTP pool and P_i, which may be the important parameter in terms of overall energy balance. The uncertainty of the NMR concentration of NTP suggests that relative changes in the rate of ATP synthetase, resulting from perturbation of cerebral energy metabolism, may prove more useful than absolute values.

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