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Kinetic analysis of the cerebral creatine kinase reaction under hypoxic and hypoglycaemic conditions *in vitro*

A ^{31}P-n.m.r. study

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1. The tissue concentration of phosphocreatine (PCr) and the pseudo-first-order rate constant of creatine kinase (k_f) were monitored in superfused guinea-pig brain slices *in vitro* by using ^{31}P-n.m.r. techniques. 2. Superfusion of slices in low oxygen partial pressure (pO_2 approx. 16 kPa) decreased tissue PCr concentrations by 48% but ATP concentrations were unchanged. Regression analysis revealed a significant negative correlation between the PCr concentration in hypoxic tissue and the increase in the rate constant, k_f. Nevertheless the forward flux through the enzyme ($J_f = k_f \cdot [PCr]$) declined under these conditions. 3. Lowering the glucose concentration to 0.2 or 0.1 mM decreased PCr concentrations by 29% and 48% respectively; here ATP concentrations as well as PCr concentrations also decreased. Only in the presence of the lower glucose concentration (0.1 mM) was k_f increased. However, unlike the situation in hypoxic tissue, J_f was maintained at control rates. 4. In spectra obtained in the presence of low oxygen or low glucose concentrations, a resonance attributable to tissue inorganic phosphate became dectectable. This observation is discussed in terms of known changes in tissue phosphate concentrations and possible alterations in cytoplasmic pH.

INTRODUCTION

The effects of moderate hypoglycaemia and hypoxia on tissue concentrations of labile phosphates such as phosphocreatine (PCr) and ATP are well characterized (Bachelard *et al.*, 1974; Lewis *et al.*, 1974; Siesjö, 1978; Cox *et al.*, 1983; Prichard *et al.*, 1983; Behar *et al.*, 1985). Nevertheless it is still unclear why mild degrees of lowered availability of glucose or oxygen should perturb physiological function in the absence of changes in overall concentrations of labile phosphates. Studies on the metabolic effects of hypoxia and hypoglycaemia have hitherto concentrated largely on monitoring steady-state concentrations of energy metabolites. Yet a greater understanding of the mechanisms of these two metabolic insults might be obtained were information available about the kinetics of energy metabolism under these conditions. Creatine kinase is an enzyme of particular interest here, in view of its central role in energy metabolism, catalysing the exchange of phosphate between ATP and PCr in excitable tissues such as brain (see McIlwain & Bachelard, 1985).

^{31}P-n.m.r. has proved to be a useful technique in studying tissue energy metabolism. Firstly, it allows continuous non-invasive monitoring of a number of key metabolites simultaneously under varying conditions. Secondly, it permits measurement of rates of enzyme-mediated exchange between these metabolites (Gadian, 1982). Such types of study have been performed on cerebral tissue, both *in vivo* and *in vitro* (see, for instance, Prichard *et al.*, 1983; Behar *et al.*, 1985; Cox *et al.*, 1983; Morris *et al.*, 1985). A previous report described the application of the n.m.r. technique of saturation transfer to studying the forward and reverse rates of creatine kinase under normal and mildly hypoglycaemic conditions (Morris *et al.*, 1985). The present paper extends that study to hypoxia and more profound degrees of hypoglycaemia.

METHODS

Tissue preparation

Adult male Dunkin–Hartley guinea pigs (250–350 g body wt.) were stunned and exsanguinated, and the brains were quickly removed. The subcortical tissues were excised, each cerebral hemisphere was cut in half with a scalpel and the two halves were sliced at right-angles to this cut in one direction, to give slices of 350 μm thickness (McIlwain & Bachelard, 1985). Immediately the slices had been prepared, they were transferred to a test tube containing gassed incubation medium, briefly dispersed with a Vortex mixer and decanted into a conical flask containing incubation medium constantly gassed with O_2/CO_2 (19:1). The froth that had accumulated was aspirated away, and the slices were then rinsed five times with fresh gassed medium. They were transferred into a standard 25 mm-diameter n.m.r. tube fitted with a polytetrafluoroethylene (PTFE) insert carrying superfusion tubing and the shaft of a motor-driven glass stirrer (Cox *et al.*, 1983). The slices were prepared at room temperature before superfusion at

Abbreviations used: PCr, phosphocreatine; FID, free induction decay.

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37 °C. The cerebral cortices from four guinea pigs were required to provide a sufficient amount of tissue for each n.m.r. experiment. The incubation medium contained NaCl (124 mM), KCl (5 mM), KH_2PO_4 (1.2 mM), $MgSO_4$ (1.2 mM), $CaCl_2$ (1.2 mM) and $NaHCO_3$ (26 mM); 10 mM-glucose was present for tissue preparation and control experiments. Gassing was with O_2/CO_2 (19:1), except during hypoxic experiments, where a gas mixture of air/CO_2 (19:1) was used. The tissues within the n.m.r. tube were superfused with medium at rates of 23–47 ml/min at 37 °C. The flow rate was adjusted to ensure continuous gentle agitation of the tissues before the tube was placed inside the n.m.r. probe (Bachelard *et al.*, 1985).

N.m.r. spectroscopy

N.m.r. spectra were obtained with a Bruker WM 200 wide-bore spectrometer, operating at a frequency of 81 MHz for phosphorus, without a deuterium lock. Magnet shimming was accomplished by using the free induction decay (FID) from the protons in the water of the circulation perfusate. A linewidth of 5–10 Hz was normally achieved. Spectra were usually accumulated in blocks of 128 × 8 k FIDs. The spectral width was 5.55 kHz. Each block was individually recorded on magnetic disc for subsequent inspection and analysis. In experiments where the contents of the superfusing medium were varied, the medium was changed without disturbing the probe (Cox *et al.*, 1983). Data collection was resumed after 22 min superfusion with altered medium.

Saturation transfer conditions

In the case of the creatine kinase equilibrium:

$$\text{Phosphocreatine} + \text{MgADP} + \text{H}^+ \rightleftharpoons \text{creatine} + \text{MgATP}$$

the forward flux can be expressed as $k_f \cdot [\text{PCr}]$, where k_f is the pseudo-first-order rate constant and [PCr] is the concentration of phosphocreatine. For determinations of k_f the γ-ATP resonance was pre-saturated for end-point times of 5 ms or 8 s, and data were acquired for a period of 0.68 s following a 90° pulse. The inter-pulse interval was 8 s plus the saturation time.

In experiments where the forward rate constants were determined under low-glucose or hypoxic conditions, data were collected in interleaved sub-blocks of eight scans and were preceded and followed by end-point determinations in control media on the same tissue preparation (for details see Morris *et al.*, 1985).

The 'γ-ATP' peak includes resonance of the γ-phosphate groups of nucleoside triphosphates and the β-phosphate groups of nucleoside diphosphates (Gadian 1982). However, more than 90% of the intensity of the 'γ-ATP' resonance is attributable to ATP (Morris *et al.*, 1985).

RESULTS AND DISCUSSION

After a 20 min period of superfusion, sufficient to ensure optimal post-preparative recovery of tissue phosphates, the ratio of the area of the PCr resonance to that of the γ-ATP resonance was found to be 1.51 ± 0.26 (means ± S.D.; $n = 5$). This is lower than the previously published value of 2.3, derived from a single experiment (Cox *et al.* 1983), but is similar to the ratios reported for the brain *in vivo* (Sauter & Rudin, 1987). Determination of the control pseudo-first-order forward rate constant of creatine kinase (k_f) gave a value of $0.22 \pm 0.05\ s^{-1}$ ($n = 16$), identical with that reported previously (Morris *et al.*, 1985). A value of the intrinsic T_1 for PCr of 4.18 s ($n = 2$; range 4.14–4.22) was derived in these earlier saturation transfer studies (Morris *et al.*, 1985).

Effects of hypoxia and hypoglycaemia

Where slices were superfused with test media, the 51 min period during which FIDs were acquired for estimation of k_f was preceded by a 22 min equilibration period. Superfusion of tissue slices with hypoxic medium decreased the amplitude of the PCr peak to 52 ± 16% of control (mean ± S.D, $n = 6$; Figs. 1*a* and 1*c*). Under

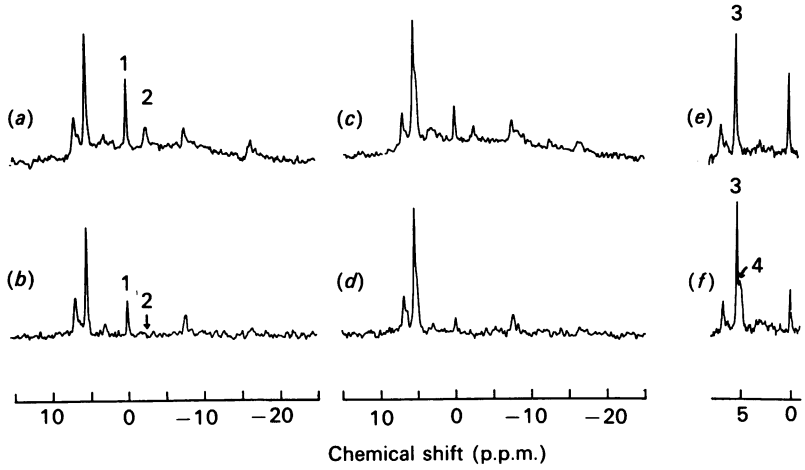

Fig. 1. Determination of forward rate constant, k_f, of creatine kinase in hypoxic tissue

(*a*) and (*b*) Spectra of cerebral tissue superfused in control medium containing 10 mM-glucose and equilibrated with O_2/CO_2 (19:1) obtained after irradiation of the tissue for 0.005 s (*a*) or 8 s (*b*) at the resonant frequency of the γ-ATP peak 2; the PCr resonance is peak 1. (*c*) and (*d*) Spectra obtained in medium equilibrated with air/CO_2 (19:1) after irradiation for 0.005 s (*c*) or 8 s (*d*). (*e*) and (*f*) Expansions of parts of spectra (*a*) and (*c*) respectively, with 12 Hz line-broadening decreased to 5 Hz to show partial resolution of the resonance attributed to tissue P_i, peak 4, just upfield of that due to medium P_i, peak 3.

Table 1. Effects of hypoxia or low glucose concentrations on PCr concentrations, k_f and J_f

Slices were superfused with control medium, and a forward rate constant was determined by saturation transfer (see the Methods section). They were then superfused with the test media as indicated for a 22 min equilibration period, before a further 51 min period during which time a test rate constant was determined (expressed here as a percentage of the control rate). PCr concentrations were determined under control and test conditions, from the amplitudes of the PCr resonances obtained in spectra where the γ-ATP resonance was irradiated for only 5 ms (a period of irradiation insufficient to saturate significantly the magnetization of the γ-ATP resonance). A test flux was calculated from the product of the test [PCr] and the test rate constant, and expressed as a percentage of the control flux. Results significantly different from control values: $*P < 0.025$; $**P < 0.010$; $\dagger P < 0.005$.

Condition	n	[PCr] (% of control)	Rate constant k_f Control (s^{-1})	Rate constant k_f Test (% of control)	Test flux J_f (% of control)
Air/CO_2 (19:1)	6	52±16†	0.22±0.05	150±70	70±19**
0.2 mM-Glucose	4	71±6†	0.25±0.04	102±17	73±17*
0.1 mM-Glucose	6	52±6†	0.19±0.05	195±76*	103±46

these conditions the pseudo-first-order rate constant, k_f, increased to 150±70% of control, although this latter effect was not statistically significant (Table 1). However, a regression analysis of the relationship between the values of k_f and the concentration of PCr for each experiment under hypoxic conditions revealed a significant negative correlation between these two parameters ($r = -0.82$; $P < 0.025$). Thus the more profound was the decrease in PCr concentration the greater was the increase in k_f (Fig. 2). Nevertheless, despite this increase in k_f, the forward flux ($k_f \cdot$[PCr]) through creatine kinase declined under hypoxic conditions.

Normally, it is difficult to detect free tissue inorganic phosphate (P_i) in n.m.r. spectra from superfused brain slices, because the quantity of free tissue P_i is small in relation to the larger amount of P_i in the superfusion medium. [The P_i in the medium is considered desirable to ensure optimal energy metabolism *in vitro* (McIlwain & Bachelard, 1985)]. However, in hypoxic tissue the P_i peak linewidth broadened, and in many cases a distinct shoulder appeared on the downfield side of the P_i resonance (Figs. 1*a*, 1*c*, 1*e* and 1*f*). Total tissue P_i concentrations rise considerably in hypoxic tissue (Siesjö, 1978; Prichard *et al.*, 1983), and thus the amount of free P_i is likely to increase to such an extent that it becomes detectable in the n.m.r. spectrum. In addition, tissue pH is known to decrease in hypoxia (Bachelard *et al.*, 1974; Prichard *et al.*, 1983; Hilberman *et al.* 1984), which would be apparent in a ^{31}P-n.m.r. spectrum as a downfield shift in the tissue P_i resonance (Gadian, 1982). The chemical shift of the tissue P_i in Fig. 1(*f*) (as indicated by the shoulder in the P_i resonance) is equivalent to a pH value of 7.0 (Gadian, 1982). The pH of our circulating buffer is approx. pH 7.3, considered to approximate the intracellular cytoplasmic pH under normal conditions *in vitro* (Lipton & Whittingham, 1984). Thus the appearance of a P_i resonance downfield from the resonance of the buffer P_i is consistent with an acidosis associated with hypoxic conditions.

In another series of experiments slices were superfused with media containing either 0.2 mM- or 0.1 mM-glucose. Both concentrations of glucose were associated with significant decreases in control concentrations of PCr (down to 71±6%, $n = 4$, and 52±6%, $n = 6$, respectively). However, only in the case of the lower concentration of glucose was any change in k_f observed: it increased to 195±76% of control ($n = 6$), significant at $P < 0.025$ (Table 1). In contrast with hypoxic tissue, the foward flux was maintained at control values in tissue superfused with 0.1 mM-glucose.

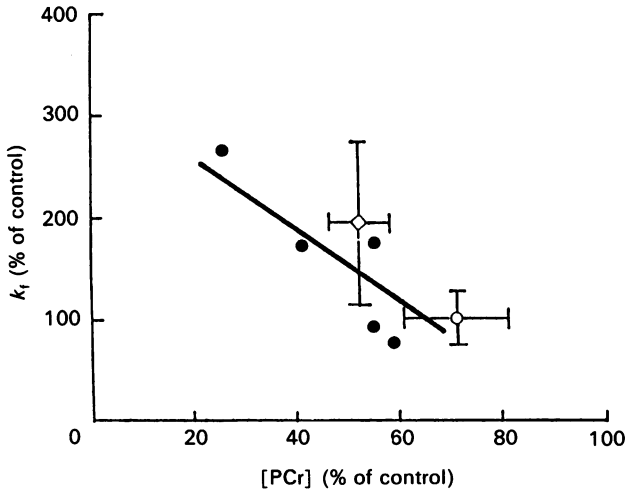

Fig. 2. Effect of hypoxia and hypoglycaemia on k_f

Values for k_f obtained under hypoxic (●) or hypoglycaemic (◇, ○) conditions are plotted against corresponding values for the concentrations of PCr. Data are expressed as percentages of control values determined in normoxic and normoglycaemic media. Individual data points and a regression line are shown for data from tissue superfused with medium equilibrated with air/CO_2 (19:1). Data for tissue superfused in media containing 0.2 mM-glucose (○) and 0.1 mM-glucose (◇) have been pooled and are shown as means±95% confidence limits.

In spectra obtained in the presence of this lower concentration of glucose, a marked shoulder appeared on the downfield side of the medium P_i resonance such that the total intensity due to P_i (tissue+medium) increased (Figs. 3*e* and 3*f*). Thus, as argued above, it seems under these conditions tissue P_i is increased. This is consistent with the increase in total tissue P_i that is known to occur in hypoglycaemic tissue (Siesjö, 1978; Prichard *et al.*, 1983; Behar *et al.*, 1985). Further, as

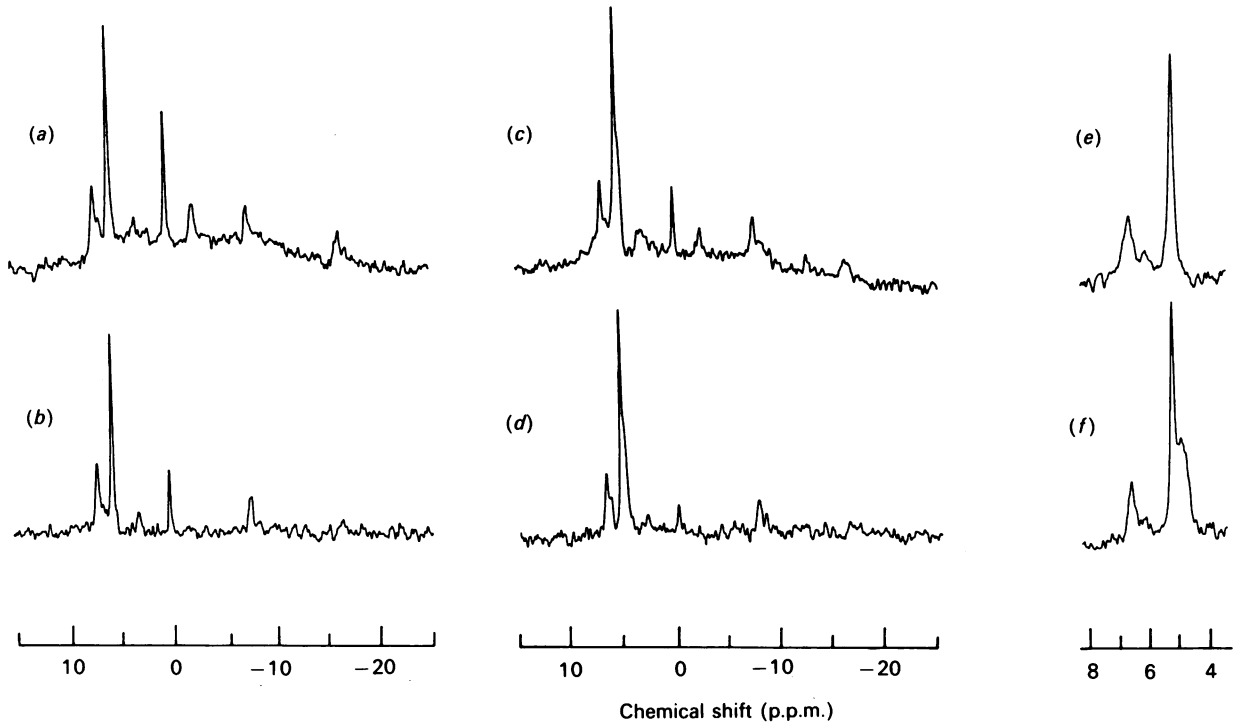

Fig. 3. Determination of forward rate constant, k_f, of creatine kinase in hypoglycaemic tissue

(*a*) and (*b*) Spectra obtained in control medium contaning 10 mM-glucose and equilibrated with O_2/CO_2 (19:1) after irradiation of the γ-ATP peak for 0.005 s (*a*) or 8 s (*b*). (*c*) and (*d*) Spectra obtained from slices superfused in medium containing 0.1 mM-glucose. In this experiment the creatine kinase rate constant was so high that the intensity of the PCr peak was barely measurable. (*e*) and (*f*) Expansions of parts of spectra (*a*) and (*c*) respectively, with 12 Hz line-broadening decreased to 5 Hz, as in Fig. 1.

discussed above for hypoxic conditions, the chemical shift of the P_i resonance in Fig. 3(*f*) is equivalent to a pH value of 7.05, thus suggesting that some tissue acidosis is occurring also in hypoglycaemic conditions in these experiments. There is considerable disagreement in the literature about the effects of hypoglycaemia on brain tissue pH. Calculations based on tissue CO_2 give pH changes *in vivo*, under conditions of isoelectric electroencephalography, between +0.15 and −0.08 unit (Lewis *et al.*, 1974; Feise *et al.*, 1977; Pelligrino *et al.*, 1981). Nor are values derived from the chemical shift of tissue P_i in brain n.m.r. spectra *in vivo* any more consistent, ranging from +0.20 to −0.07 (Prichard *et al.*, 1983; Behar *et al.*, 1985). Resolution of this contradiction in n.m.r. experiments awaits the application of unambiguous measures of cytoplasmic pH, such as spectroscopy of fluorine-labelled pH indicators, or of titratable species, especially protons (Deutsch *et al.*, 1982).

In 12 experiments the 51 min superfusion with test media was immediately followed by superfusion of the slice with control medium. Under these conditions k_f was found to be $0.21 \pm 0.05\ s^{-1}$, a value indistinguishable from that obtained before superfusion with test medium.

Changes in brain PCr and ATP concentrations under hypoxic and hypoglycaemic conditions have been well characterized both by n.m.r. techniques (Prichard *et al.*, 1983; Cox *et al.*, 1983; Bachelard *et al.*, 1985; Behar *et al.*, 1985) and by enzymic methods (see Siesjö, 1978). It is clear from these results that the concentrations of the two labile phosphates fall together if glucose availability is restricted, but only PCr concentrations decline under moderately hypoxic conditions. As far as we are aware, this is the first report on the effects of controlled hypoxia on the kinetics of cerebral creatine kinase; a preliminary study on hypoglycaemic cerebral tissue was previously described (Morris *et al.*, 1985).

The results of our kinetic investigation show that, although the forward rate constant of the creatine kinase reaction increases under hypoxic and profoundly hypoglycaemic conditions, only in the latter condition is the forward flux through the enzyme maintained at control values. The forward flux is equal to the product of the forward rate constant and the concentration of PCr, i.e. $J_f = k_f \cdot [PCr]$. Consequently, if J_f is to be maintained while [PCr] is falling, profound hypoglycaemic conditions must in some way be leading to a compensatory increase in k_f. Under hypoxic conditions k_f is increasing, but not sufficiently to counteract the fall in [PCr].

How then does hypoglycaemia lead to a greater increase in k_f than does hypoxia, for a given decrease in [PCr]? One significant difference between the two metabolic insults was referred to above, namely that in moderate hypoxia only PCr concentrations fall whereas in hypoglycaemia both PCr and ATP concentrations decline in parallel. Should creatine kinase remain at equilibrium under the various conditions used here, the foregoing would imply that increases in ADP are greater in hypoglycaemia than in hypoxia, thus contributing to the greater increase in k_f. Indeed, Matthews *et al.* (1982) have argued that in the heart the free cytosolic ADP is

primarily responsible for regulating the forward flux of creatine kinase.

A further difference between the two conditions is that whereas increased lactic acid production occurs in hypoxia, it does not occur in hypoglycaemia. However, whether or not changes in pH take place in hypoglycaemia is still equivocal, as noted above. The results described here suggest that pH may fall under both conditions, although these observations require confirmation and are at present qualitative rather than quantitative.

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