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The utilisation of creatine and its analogues by cytosolic and mitochondrial creatine kinase

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Abstract

We have investigated the utilisation of four analogues of creatine by cytosolic Creatine Kinase (CK), using ^{31}P-NMR in the porcine carotid artery, and by mitochondrial CK (Mt-CK), using oxygen consumption studies in isolated heart mitochondria and skinned fibres. Porcine carotid arteries were superfused for 12 h with Krebs-Henseleit buffer at 22°C, containing 11 mM glucose as substrate, and supplemented with either 20 mM β-guanidinopropionic acid (β-GPA), methyl-guanidinopropionic acid (m-GPA), guanidinoacetic acid (GA) or cyclocreatine (cCr). All four analogues entered the tissue and became phosphorylated by CK as seen by ^{31}P-NMR. Inhibition of oxidative metabolism by 1 mM cyanide after accumulation of the phosphorylated analogue resulted in the utilisation of PCr, β-GPA-P, GA-P and GA-P over a similar time course (~ 2 h), despite very different kinetic properties of these analogues in vitro. cCr-P was utilised at a significantly slower rate, but was rapidly dephosphorylated in the presence of both 1 mM iodoacetate and cyanide (to inhibit both glycolysis and oxidative metabolism respectively). The technique of creatine stimulated respiration was used to investigate the phosphorylation of the analogues by Mt-CK. Isolated mitochondria were subjected to increasing [ATP], whereas skinned fibres received a similar protocol with increasing [ADP]. There was a significant stimulation of respiration by creatine and cCr in isolated mitochondria (decreased K_m and increased V_{max} vs control), but none by GA, mGPA or β-GPA (also in skinned fibres), indicating that these latter analogues were not utilised by Mt-CK. These results demonstrate differences in the phosphorylation and dephosphorylation of creatine and its analogues by cytosolic CK and Mt-CK in vivo and in vitro.

Keywords: Creatine kinase; Creatine analogue; Creatine-stimulated respiration; Mitochondrion; Nuclear magnetic resonance; Porcine carotid artery

1. Introduction

Creatine is phosphorylated by creatine kinase (CK) to produce phosphocreatine (PCr). The existence of this CK system has been well-documented in many mammalian tissues, but its exact role and importance in cellular energetics are still a matter of discussion [1].

In tissues with high and fluctuating energy demands, such as muscle and brain, ATP can be replenished with PCr via the CK reaction. The CK system is thought to either buffer changes in the ATP concentration, or to shuttle high-energy phosphate groups from areas of ATP production (e.g., mitochondria) to areas of ATP consumption (e.g., contractile proteins) [1,2]. Much evidence for this latter hypothesis comes from the existence and subcellular localisation of CK isoenzymes [2].

The five isoenzymes of CK currently known to exist in mammalian tissues, originate from four gene products [2]. Three isoenzymes are found in the cytoplasm, and two in the mitochondria. The cytosolic isoenzymes form dimers from the two monomers, M (muscle type) and B (brain type). The MM isoenzyme is found predominantly in skeletal muscle and heart; BB is found in smooth muscle and neural tissue; and MB is found in heart, and developing skeletal muscle.

The two mitochondrial isoenzymes are both found in the intermembrane space, either free or bound to the inner mitochondrial membrane [3,4]. In vitro, mitochondrial CK (Mt-CK) has been shown to form stable dimeric and octameric molecules which are readily interconvertible. Electron microscopy has shown that the octameric forms

Abbreviations: CK, creatine kinase; Cr, creatine (*N*-methyl(glycocyamine)); PCr, phosphocreatine (*N*(-phosphonoamidino)sarcosine); GA, guanidinoacetic acid (glycocyamine); β-GPA, β-guanidinopropionic acid (*N*-amidino-β-alanine); cCr, cyclocreatine (1-carboxymethyl-2-iminoimidazolidine); mGPA, methyl-guanidinopropionic acid (*N*,*N*-methylamidino-β-alanine); Mt-CK, ubiquitous mitochondrial creatine kinase.

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of Mt-CK display a very similar structure in all tissues so far studied [3], appearing cube-like, with four-fold symmetry and a central cavity. Octameric Mt-CK is thought to be the prominent form in vivo.

Mt-CK phosphorylates creatine, and has been suggested to have privileged access to mitochondrial (oxidative) ATP, which is transported by the adenine nucleotide translocase (ANT). The ADP produced is transported back into the mitochondrial matrix via the ANT, whilst PCr passes into the cytoplasm. The resulting ADP can stimulate oxidative phosphorylation. The overall process by which the phosphorylation of creatine can lead to a stimulation of oxidative phosphorylation is known as *creatine-stimulated respiration* [5,6].

Attempts to assess the function and importance of the creatine kinase system in cellular energetics have used creatine analogues such as β-guanidinopropionic acid (β-GPA) to deplete creatine and phosphocreatine in the cell [7–14]. β-GPA competes with creatine for uptake into cells [15,16] and is a poor substrate for CK (increased Michaelis constant and decreased maximum velocity) [14]. Animals fed for 5–19 weeks on a 1% diet of β-GPA show a 90% reduction of PCr in heart and skeletal muscle and replacement with β-GPA-P [7,8,11–13]. Depletion of the creatine pool in this way, however, is associated with modifications (pathological or adaptive) that may be due to an altered energetic status in the cell [4,12,13,17]. In particular, a proliferation of Mt-CK was observed in β-GPA fed animals which was attributed to a lowered energy state in the cells [10]. As β-GPA is not a very effective substrate for Mt-CK [2,3], based on in vitro kinetic studies using purified MM CK [18], it was thought that Mt-CK could not act on or respond to β-GPA due to some specificity of Mt-CK [4,19]. This proved to be correct, and it was concluded that it was the low concentration of substrate for Mt-CK (and not the energy state of the cell) that signalled Mt-CK proliferation [4,19]. The reason for the difference between creatine and β-GPA was, however, not addressed.

Further studies have shown that the feeding of other creatine analogues results in replacement of tissue PCr pools by synthetic phosphagens with different kinetic and thermodynamic properties. Consequent subtle changes in intracellular energy metabolism [9,20] appear to have potential therapeutic effects. In particular, cyclocreatine, which as a poor phosphate donor has been shown to have beneficial effects in cancer chemotherapy [21–24]. Similarly, the same analogue has shown anti-viral properties [23], delayed onset of rigor in ischaemic hearts [25] and protective energetic effects in brain [26] and skeletal muscle [27]. In vitro predictions for analogue utilisation by CK in vivo, do not differentiate between cytosolic and Mt-CK. Indeed, in vitro kinetics from studies on purified MM-CK seem to predict very different properties to those seen in vivo, and this may be related to the different specificities of cytosolic CK compared with Mt-CK. The differential utilisation of creatine and its analogues in vivo by either cytosolic or Mt-CK has not been investigated. A possible difference in specificity may have relevance in explaining the in vivo effects of creatine analogues, and may help to explain potential therapeutic properties of other analogues.

This study was undertaken to elucidate further the specificity of CK isoenzymes for creatine and some structural analogues of creatine. We used the porcine carotid artery (containing > 95% cytosolic CK) [28] to study the utilisation of creatine and its analogues by cytosolic CK (using ^{31}P-NMR) and rat heart mitochondria to study the phosphorylation of creatine and its analogues by Mt-CK (using oxygen consumption studies). In this way, we have examined and compared the specificity of Mt-CK and cytosolic CK and some of the structure–function relationships of creatine and its analogues.

2. Materials and methods

2.1. Porcine carotid arteries

2.1.1. Tissue isolation

Porcine carotid arteries were collected at the abattoir in ice-cold physiological saline solution (PSS) containing (in mM) 116 NaCl, 25 $NaHCO_3$, 5.4 KCl, 5 KH_2PO_4, 1.2 $CaCl_2$, 1.25 $MgSO_4$, and 11 glucose. The arteries were debrided of fat and connective tissue [29] before the experiments.

2.1.2. Perfusion

One artery was chosen and placed in a 5-mm diameter tube. This was superfused (at 22°C and a constant flow rate of 15 ml/min) with PSS containing 0.5 mM KH_2PO_4 and bubbled with 95% O_2–5% CO_2. Perfusate was siphoned off from just above the carotids and recirculated at a constant volume of 5 litres. Once inside the magnet, the carotids were perfused for ~ 90 min in control media, then in the presence of PSS supplemented with 20 mM β-GPA, GA, mGPA or cCr.

In cyanide perfusion experiments, 1 mM cyanide was added to the PSS after significant phosphorylation (≥ 100% of the PCr concentration, as determined by peak height) of the analogue was observed in the NMR.

2.1.3. ^{31}P-NMR

The carotids were equilibrated for ~ 20 min in the NMR tube outside of the magnet. The tube was then loaded into a wide-bore (72 mm) 9.6-T vertical magnet using a home-built NMR probe interfaced with a Bruker Biospec spectrometer. The homogeneity of the magnetic field was optimised by shimming on the proton resonance. ^{31}P-NMR spectra were acquired at 161.97 MHz, using a 60° pulse width of 9 ms and an interpulse delay of 2.15 s. Free induction decays were Fourier transformed using exponential line broadening of between 10 and 20 Hz

depending on the analogue used (due to different chemical shifts of the phosphorylated analogues). A spectral width of 8000 Hz was used with 2048 data points. Up to 800 FIDs were summed for each spectrum. Control spectra were acquired for ~ 2 h to show the tissue stability, then for up to 24 h with the respective experimental buffer. Spectra were quantified by integration of peaks, fitted to a Lorentzian line shape using NMR1 software.

2.2. Rat heart

2.2.1. Heart preparation

Rats were sacrificed by cervical dislocation and their hearts rapidly removed and rinsed in cold (4°C) PSS buffer. These were either placed in skinning solution or isolation medium (see below).

2.2.2. Isolation of mitochondria

Rat heart mitochondria were isolated by a trypsin digestion procedure [30]. Hearts were finely minced with surgical scissors in a solution of 0.3 M sucrose, 10 mM Na-Hepes and 0.2 mM EDTA (pH 7.2) at 4°C (isolation medium). The mince was incubated for 15 min with 125 mg/ml trypsin (Fluka) and neutralised by excess trypsin inhibitor (Fluka) before gentle homogenisation with a glass Teflon homogeniser. The homogenate was centrifuged at $1000 \times g$ for 10 min and the pellet discarded. The supernatant was centrifuged at $8000 \times g$ for 15 min. The mitochondria were resuspended in isolation medium containing 1.0 mg/ml fatty acid free-BSA and the centrifugation was repeated.

2.2.3. Fibre preparation

Endocardial fibre bundles with a diameter of approx. 300 mm were isolated from the left ventricle of freshly excised rat heart and transferred into skinning solution (S, see below) as described by Veksler et al. [31]. Bundles were incubated with intense stirring for 20 min in solution S containing 40 μg/ml saponin. Bundles were then washed for 10 min in respiration solution (R, see below) without high energy phosphates at 4°C. Using saponin (40 μg/ml), the plasma membrane is selectively permeabilised such

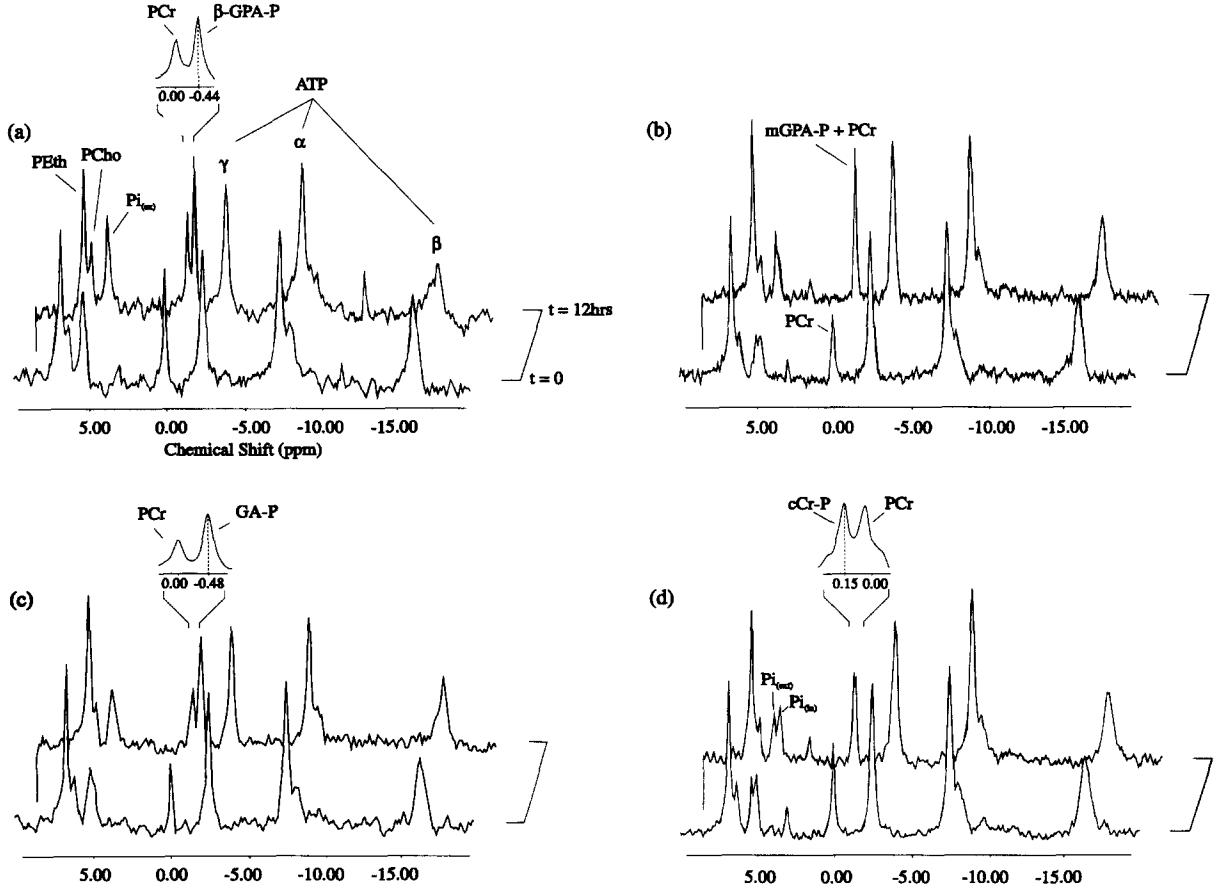

Fig. 1. Stack plot of [^{31}P]nuclear magnetic resonance (NMR) spectra showing the effects of 12 h perfusion with 20 mM (a) β-guanidinopropionic acid (β-GPA), (b) methyl-guanidinopropionic acid (mGPA), (c) guanidinoacetic acid (GA) and (d) cyclocreatine (cCr) on porcine carotid artery in the presence of 11 mM glucose. Spectra at $t = 0$ are control spectra at the start of perfusions. Peak positions are as indicated. PEth, phosphoethanolamine; PChl, phosphocholine; PCr, phosphocreatine; mGPA-P, phosphorylated mGPA; β-GPA-P, phosphorylated β-GPA; GA-P, phosphorylated GA; cCr-P, phosphorylated cCr.

that the mitochondrial function can be determined for mitochondria that have not experienced the rigorous isolation procedure. Respiratory parameters in saponin skinned fibres were determined using the method described previously for heart muscle [31].

2.2.4. Measurement of respiratory parameter

The respiratory rates were determined by a Clark oxygen electrode (Hansatech) in an oxygraph containing a mitochondrial suspension, or 2–5 mg dry weight of fibres, in solution R at 30°C with continuous stirring. The solubility of oxygen was taken to be 400 ng atoms oxygen/ml. Fibres or mitochondria were introduced into the oxygraph cell and oxygen consumption measured during sequential substrate addition. Fibres were dried and weighed, with respiration rates expressed as ng atoms of oxygen/min/mg d.w. for the fibres. Mitochondria were assayed for protein content by the Biorad assay and results reported as ng atoms of oxygen/min/mg soluble protein for mitochondria.

2.2.5. Kinetics

ATP kinetic experiments were performed on isolated rat heart mitochondria. Mitochondria were introduced into the oxygraph cell and exposed to increasing [ATP] in the presence (20 mM) and absence of creatine and its analogues. The ATP stimulated respiration above basal oxygen consumption was used to determine the apparent K_m and V_{max} of the mitochondria for ATP, in the presence of Cr, GA, mGPA and β-GPA. Other experiments on rat heart fibres were done by addition of ADP to determine the apparent K_m and V_{max} in the presence (20 mM) and absence of creatine and its analogues.

2.2.6. Solutions

Skinning solution (S) and Respiration solution R contained (mM) EGTA 10 (pCa^{2+} 7), free Mg^{2+} 3, taurine 20, dithiothreitol 0.5, and imidazole 20 (pH 7.0). Ionic strength was adjusted to 0.16 M by addition of potassium 2-(N-morpholino)ethanesulfonate. Solution S also contained 5 mM MgATP and 15 mM PCr. In place of high energy phosphates, solution R contained 5 mM glutamate, 2 mM malate, 3 mM phosphate and 2 mg/ml fatty acid free BSA [4].

2.2.7. Materials

β-GPA, cyclocreatine and mGPA were synthesised in the laboratory using the methods of Rowley et al. [18]. Purity was checked by ^{1}H-NMR. Raw materials were purchased from Apin Chemicals Ltd.

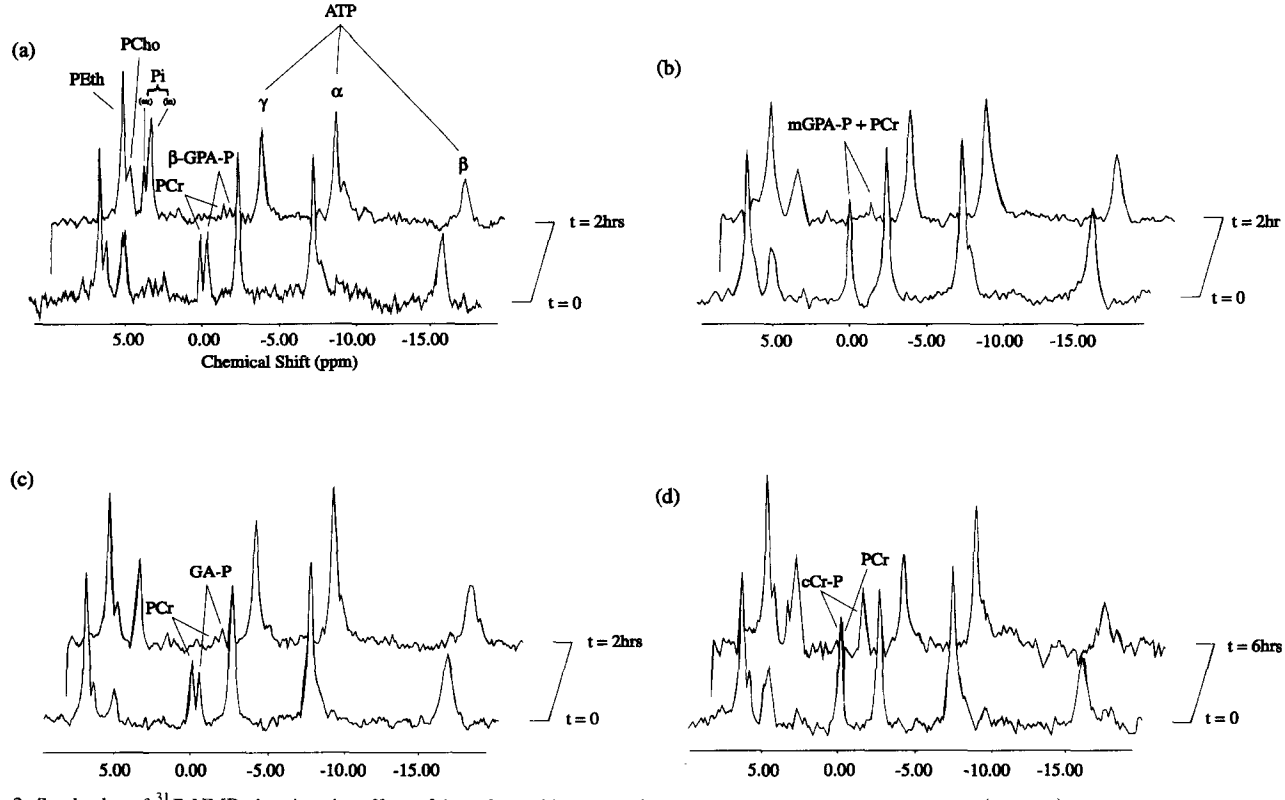

Fig. 2. Stack plot of ^{31}P-NMR showing the effect of 1 mM cyanide on porcine carotid arteries, after accumulation (at $t = 0$) of greater than 1:1, PCr: (a) β-GPA-P, (b) mGPA-P, (c) GA-P and (d) cCr-P, in the presence of 11 mM glucose. PCr, β-GPA-P, mGPA-P and GA-P are all utilised within 2 h, whereas cCr-P persists. Abbreviations are as in Fig. 1.

2.2.8. Statistics

The test for significance was the Student's *t*-test. Results are reported as standard error of the mean.

3. Results

3.1. NMR

3.1.1. Phosphorylation

Fig. 1 shows ^{31}P-NMR spectra from porcine carotid arteries perfused with 20 mM (a) β-GPA, (b) mGPA, (c) GA, or (d) cCr. Perfusion with each analogue led to the appearance of the phosphorylated analogue after a similar lag period of ~2–3 h (not shown), with a significant accumulation (> [PCr]) of the phosphorylated analogue after 12 h in all groups. The resonances for the phosphorylated analogues are (ppm): β-GPA, −0.44; GA, −0.48; cCr, +0.15; mGPA, co-resonant with phosphocreatine. The accumulation of each phosphorylated analogue was not significantly different after 12 h ($n \geq 3$), as determined from the respective peak areas.

3.1.2. Dephosphorylation

In a separate set of experiments, 1 mM cyanide was added to the perfusion buffer when the P-analogue: PCr ratio was ~1:1 (determined for mGPA when the combined mGPA/PCr peak was greater than twice the PCr peak at $t = 0$). The effects of cyanide on both PCr and phosphorylated analogue is shown in Fig. 2. In the presence of cyanide the PCr, GA-P, β-GPA-P and mGPA/PCr peaks disappear after about 2 h. with a concomitant increase in intracellular phosphate. In contrast, cCr-P is still present in significant amounts after 6 h (Fig. 2d).

Fig. 3 shows the effects of adding 1 mM iodoacetate (IAA) to the perfusion buffer containing cCr after 6 h perfusion with cyanide. IAA inhibits sulfydryl group containing enzymes, and in particular, glyceraldehyde-3-phosphate dehydrogenase. It is therefore a non-specific inhibitor of glycolysis. After about 40 min, both ATP and cCr-P begin to decrease in parallel. There is a concomitant increase in the intracellular phosphate peak, and phosphomonoester peaks, possibly due to an accumulation of glycolytic intermediates such as glyceraldehyde phosphate. By 2 h, ATP and cCr-P are indistinguishable from the noise.

3.2. Rat heart

3.2.1. Isolated mitochondria

Using sequential additions of ATP, respiration is supported by ADP produced by the MgATPase reaction(s) in the mitochondria. The kinetic parameters given characterise this reaction (Table 1, control values). In the presence of creatine, as the reaction catalysed by Mt-CK is the dominant source of ADP, the V_{max} in the presence of ATP

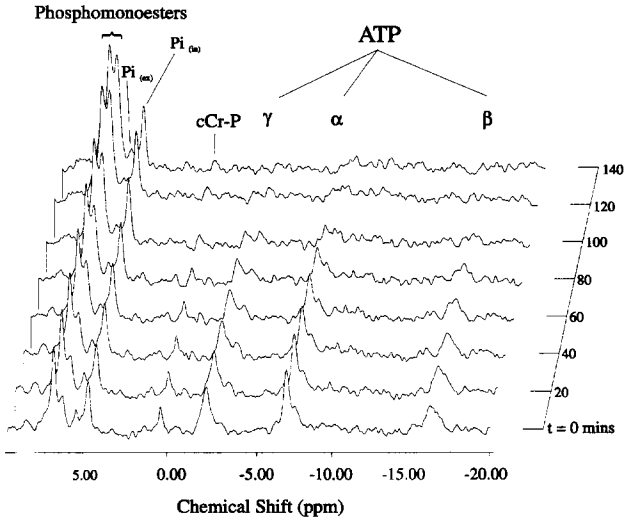

Fig. 3. Stack plot of ^{31}P-NMR showing the effect 1 mM iodoacetate (IAA). Spectrum at $t = 0$ is the spectrum from perfusion with 20 mM cyclocreatine (cCr) for 12 h (Fig. 1d) followed by perfusion with 1 mM cyanide for 6 h (Fig. 2d). Perfusion with IAA leads to the loss of both cCr-P and ATP after 2 h, with the concomitant increase in intracellular phosphate ($P_{i(in)}$) and phosphomonoesters (e.g., Glyceraldehyde-6-phosphate and other glycolytic intermediates). Abbreviations are as in Fig. 1.

is increased. An increased V_{max} associated with a decreased K_m (indicating an effective coupling of the CK reaction to oxidative metabolism [5]) is termed *creatine-stimulated respiration* [6,30,39].

Due to an increased V_{max}, and decreased K_m, in the presence of cCr, it was concluded that cCr was phosphorylated by Mt-CK. The increase in V_{max} was less than that seen in the presence of creatine, but the K_m was decreased to a greater extent (Table 1).

Neither GA, β-GPA [4], or m-GPA had any significant effect on the mitochondrial response to ATP compared with control. We conclude that there was no GA-, β-GPA-, or m-GPA-stimulated respiration and that Mt-CK was unable to phosphorylate these analogues under these conditions.

Table 1
Respiration in isolated mitochondria

Substrate	CSR	V_{max} (ng atoms O mg protein^{-1} min^{-1})	K_m (μM ATP)	*n*
None [b]	−	273±16	46.9±3.6	10
Creatine	+++	473±44 [a]	24.8±2.9 [a]	9
cCr	+	363±35 [a]	18.0±1.6 [a]	15
GA	−	−	−	4
β-GPA [b]	−	319±4	60.3±5.5	10
mGPA	−	−	−	4

± Standard error of the mean.
[a] Significantly different from control ($P < 0.05$).
CSR-creatine stimulated respiration (relative stimulation).
[b] [4].

3.2.2. Fibres

When the analogue appeared to be unable to stimulate respiration, experiments were repeated with saponin-permeabilised myocardial fibres, with creatine-stimulated-respiration observed as a decrease in apparent K_m for ADP [4,5]. There were no significant differences in the respiratory responses in the presence of GA, β-GPA [4], or m-GPA (data not shown). We conclude that Mt-CK is unable to act on or respond to these analogues.

4. Discussion

We have demonstrated previously that the creatine analogue β-GPA is phosphorylated by creatine kinase in the perfused porcine carotid artery [29]. It was concluded that β-GPA was phosphorylated by cytosolic isoforms of CK because β-GPA was not phosphorylated by Mt-CK [4,19]. Cr and β-GPA are structural isomers. Cr is methylated on its tertiary nitrogen (Fig. 4a), whilst in β-GPA, the methyl group is positioned in the carbon chain (Fig. 4d). There are two possible reasons why β-GPA is not phosphorylated by Mt-CK. Either the methyl group is important in the active site, or the carbon chain in the β-GPA molecule is too long to fit in the active site. The aim of this study was to investigate the utilisation and specificity of β-GPA and three other analogues of creatine by both cytosolic and Mt-CK, by their ability to be phosphorylated and dephosphorylated.

4.1. Cytosolic CK

4.1.1. Phosphorylation

The creatine analogues GA, β-GPA, m-GPA and cCr are all phosphorylated by creatine kinase in the porcine carotid artery, as evidenced by the appearance of NMR peaks co-resonant with, or in the vicinity of the PCr peak (Fig. 1). The accumulation of each phosphorylated analogue after 12 h was not significantly different, as determined by measuring peak areas. This is interesting when one considers the different reactivities of these analogues in vitro. Due to the high percentage of cytosolic CK in the porcine carotid artery (> 95%) [28], we assume that a large proportion of the creatine analogue is phosphorylated by cytosolic CK in vivo. Reactivity measured in vitro with rabbit muscle MM CK occurs in the order (number of fold less reactive than Cr): cCr, (30); GA, (100); mGPA, (500); and β-GPA (1500) [32]. This selectivity of CK might be explained by studying the structures of the analogues. Two main structural parameters investigated are the carbon chain length, and the geometry created by the methyl group on the tertiary nitrogen. NMR studies of cCr and cCr-P have shown that the primary amino group of this compound is phosphorylated. This result and others, strongly imply that creatine is phosphorylated on the guanidino nitrogen that is trans to the methyl group when it is bound to the enzymes surface [18]. This *N*-methyl group, which is considered an important structural feature for the CK reaction, as Cr is 100 times more reactive than GA itself, has been proposed to cause a favourable conformational change of the enzyme to occur at the active site [33]. The carbon chain length is also important as seen by the difference in reactivity measured in vitro between Cr and mGPA (Fig. 4a,b), and GA and β-GPA (Fig. 4c,d) [18,32]. Interestingly, cyclocreatine, and homocyclocreatine, which differ by a methylene group in the same way as creatine and mGPA, differ in reactivity to a similar extent [32]. Our studies have demonstrated that this order of reactivity is not apparent in vivo, most probably because the range of reactivities of the creatine analogues (30- to

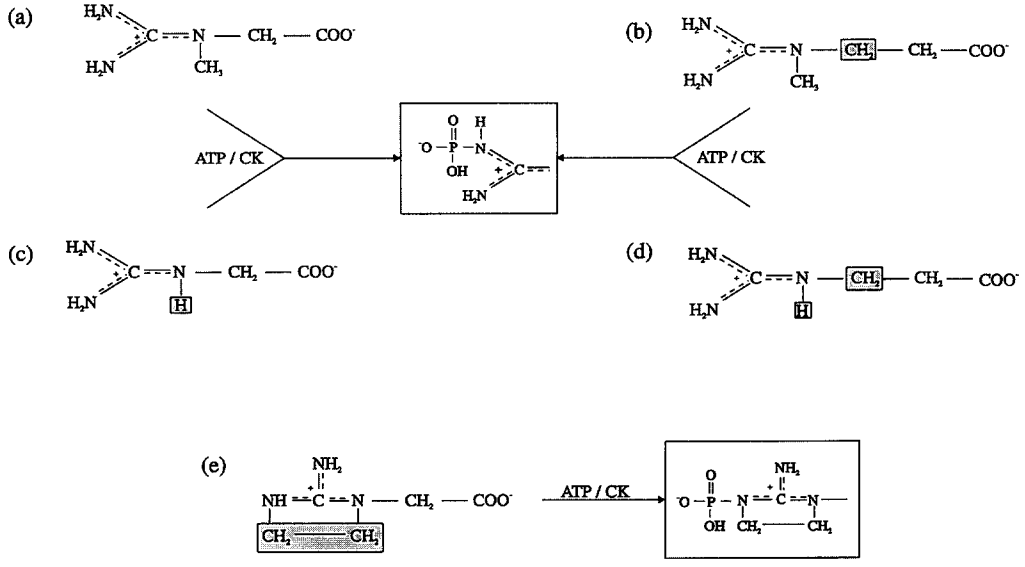

Fig. 4. Structures of (a) creatine (Cr), (b) methyl-guanidinopropionic acid (mGPA), (c) guanidinoacetic acid (GA), (d) β-guanidinopropionic acid (β-GPA) and (e) cyclocreatine (cCr), showing the relative site of phosphorylation.

1500-fold less reactive than creatine) are similar to the rate of the CK flux compared with the ATPase flux [34,35]. Interestingly, perfusion with Cr for 12 h leads to an increase in PCr that is similar in magnitude to the increase seen with perfusion with mGPA [36] i.e. even Cr as the most reactive substrate for CK still does not appear to be phosphorylated more effectively in vivo in the porcine carotid artery than any of the analogues studied, as seen by NMR. It is assumed that at a perfusion concentration of 20 mM, creatine and its analogues are flooding across the membrane, and that phosphorylation is not transport-limited.

4.1.2. Dephosphorylation

To investigate the effectiveness of the phosphorylated analogue as a substrate for CK, we added cyanide to the 'analogue loaded tissue' in order to inhibit oxidative metabolism. Oxidative metabolism in smooth muscle has been linked to PCr production, which has been shown to be important for contractile function [37,38]. In the unstimulated resting smooth muscle preparations used here, the function of PCr, and indeed oxidative metabolism is not clear. The low basal oxygen consumption in resting porcine carotid artery (JO_2 of 0.069 μmol g wet wt.$^{-1}$ min^{-1}) [40] predicts a low turnover of ATP. It can be postulated then, that in our preparations, a resultant low flux through the CK reaction leads to a small rate of production of PCr. We and others have shown that inhibition of oxidative metabolism with cyanide or hypoxia leads to a loss of PCr as seen by NMR, but only to a small decrease in ATP [29,41,42]. The contribution of glycolysis to vascular smooth muscle metabolism can be calculated under anaerobic conditions (such as in the presence of cyanide), to produce up to 70% of the resting tissue [ATP] [40]. This explains why [ATP] can be maintained under anaerobic conditions, and with the loss of PCr supports compartmentation of metabolites. The fact that β-GPA is poorly phosphorylated in the presence of cyanide (but with a maintained [ATP]), also predicts some compartmentation of metabolites in this tissue [29].

In the presence of the phosphorylated analogue, cyanide leads to the loss of GA-P, β-GPA-P and m-GPA-P within 2 h (Fig. 2a–c). No significant difference was seen in the rate of breakdown of β-GPA-P (the least reactive analogue in vitro) compared with the rate of breakdown of PCr (Fig. 5). This is surprising as β-GPA-P is a poor substrate for CK in vitro, with approx. 1/1500th the reactivity of phosphocreatine [14]. As discussed above, the CK reaction in vivo, is two-to-three orders of magnitude faster than the ATPase activity [34,35], so that using this criterion, a poor substrate for CK in vitro can again be used by CK in vivo.

cCr-P is dephosphorylated over a much slower time course than GA-P, β-GPA-P and mGPA-P, decreasing by < 50% after 6 h (Fig. 2d). This difference is reflected in the kinetics of cytosolic CK measured for cCr. Table 2a gives literature values of the K_m and V_{max} (of cytosolic CK) for Cr, β-GPA and cCr in both phosphorylated and unphosphorylated forms. Although not a definitive term, we may assume that the value given by V_{max}/K_m for an enzymatic reaction is the propensity for that reaction to proceed in a particular direction. The values calculated for the phosphorylated and unphosphorylated analogues show that both Cr and β-GPA are more likely to be dephosphorylated (as seen by the difference in V_{max}/K_m for the forward and reverse reaction, i.e., $Y_p/Y_{-p} > 10$ (Table 2a), whereas the values calculated for cCr and cCr-P imply that

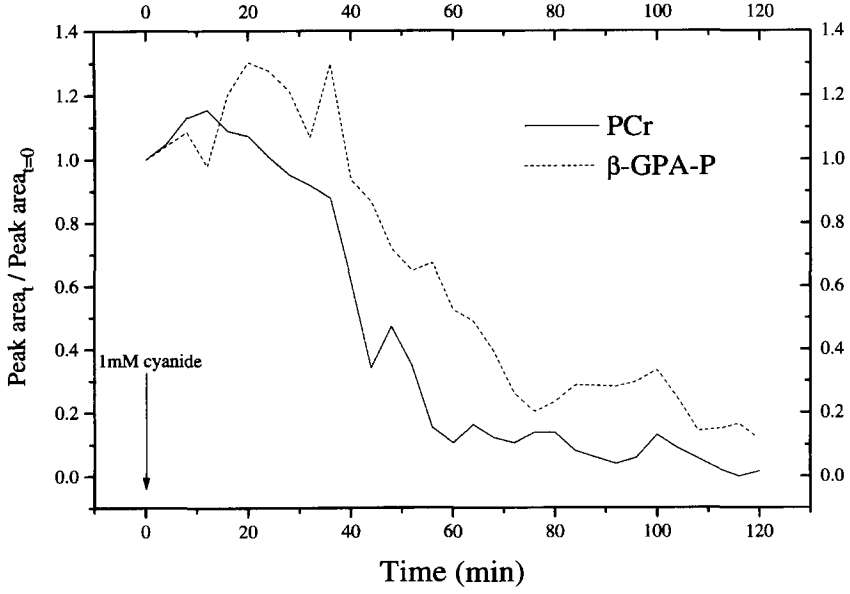

Fig. 5. Graph showing rate of decrease of PCr and β-GPA-P in the porcine carotid artery after addition of 1 mM cyanide. Metabolite concentration is represented as the peak area relative to the peak area at $t = 0$ (the point of cyanide addition).

Table 2
Kinetic data from (a) cytosolic and (b) mitochondrial creatine kinase

Substrate (cytosolic CK)	V_{max} (μM/min/ /mgCK)	K_m (mM)	V_{max}/K_m (Y)	Y_p/Y_{-p}
(a)				
Cr [a]	75.1	16.7	4.5	
PCr [a]	231	2.2	105	23.3
β-GPA [a]	0.21	50	0.0042	
β-GPA-P [a]	0.22	4.9	0.045	10.7
cCr [b]	1.88	55	0.62	
cCr-P [b]	33.9	3.7	0.50	0.8
(b)				
Substrate (Mt-CK)	V_{max} (μM/ min/mgCK)	K_m (mM)	V_{max}/K_m (Y)	Y_p/Y_{-p}
Cr [c]	15	8.0	1.88	
PCr [c]	45	3.0	15.0	7.9

[a] [14]
[b] [43]
[c] [47].
Y_p/Y_{-p}; ratio of V_{max}/K_m (phosphorylated form):V_{max}/K_m(unphosphorylated form).

the reaction has no real preference to proceed in either direction ($Y_p/Y_{-p} < 1$). Utilisation of cCr-P is subject to kinetic constraints which derived from the fact that its Gibbs energy of hydrolysis is 8.4 kJ/mol lower than that of PCr. Comparison of V_{max}/K_m ratios for cCr-CK equilibrium and Cr-CK equilibrium, obtained in vitro with purified MM CK have indicated that Cr is approximately a 6- to 7-fold better substrate for CK than cCr (McLaughlin et al. [43]) at 37°C, whereas PCr is a 200-fold better substrate than cCr-P (Table 2) [14,44]. The relative inability of CK to utilise cCr-P is demonstrated in its slow utilisation in the presence of cyanide (seen in Fig. 2d).

As the high glycolytic capacity of smooth muscle can maintain ATP in the absence of oxidative ATP, then the CK reaction appears to follow the prediction made by solution kinetics (V_{max}/K_m) (Table 2). To determine if cCr-P can be utilised by CK, we used IAA in the presence of cyanide to inhibit glycolysis as well as oxidative metabolism to create a situation where ATP is rapidly falling. Under these conditions, cCr-P and ATP both decreased at similar rates, indicating that cCr-P can indeed be utilised by CK (Fig. 3). This is in agreement with work in chronically fed animals which have shown the utilisation of cCr-P under conditions of ATP depletion [20,26,27,45–47].

PCr, β-GPA-P, GA-P and m-GPA-P all act similarly in the presence of cyanide; cCr-P behaves differently, however, which can be explained by studying its structure and kinetics. cCr is the only analogue studied which has a greater propensity to exist in its phosphorylated form in the presence of CK, as shown by in vitro kinetics and confirmed here by NMR. The difference can be attributed to its planar 5-membered ring structure (Fig. 1e) [18]. Although cCr is a relatively good substrate for CK in vitro, the puckered 6-membered ring structure of 1-carboxymethyl-2-iminohexahydropyrimidine (with an extra $-CH_2$ in the ring) is over 400 times less reactive [18]. This and other measurements indicate a very low affinity by the active site of CK for substituents which project from the plane defined by the three guanidino nitrogen atoms and suggests that portions of the active site are only accessible to relatively flat, and compact structures. It is likely that phosphorylation of cyclocreatine results in a planar-to-puckered transformation, which is reflected in V_{max}/K_m values (Table 2) [18]. Other work has shown that as these values would predict (in analogue fed animals), cCr-P can accumulate to high concentrations in the cell, due to the relative low reactivity of the phosphorylated form [20,47].

Although in vitro kinetic data on cytosolic CK predicts otherwise, in vivo feeding studies have shown that many analogues of CK can serve as potential substitutes for creatine. Our acute studies here have confirmed this, showing that even the poorest substrate studied, β-GPA/β-GPA-P, can be readily utilised in a living tissue containing $> 95\%$ cytosolic CK. Indeed, it was dephosphorylated with a rate similar to PCr, when the kinetics would predict a 1500-fold difference in rate, implying that dephosphorylation is determined by the ATPase rate and not CK. However, acute studies with cCr have shown that the predicted properties in vitro can correspond to what is seen in vivo. Care must therefore be taken if one wishes to predict in vivo kinetics using in vitro data. It is likely that the kinetic properties of cCr have relevance to this analogue's potential therapeutic properties. In cells with a high metabolic turnover, feeding creatine or an analogue might be expected to lead to a high rate of utilisation. The kinetic properties of cCr, however, would lead to a high rate of phosphorylation, but a low rate of dephosphorylation. Cyclocreatine might thus act as a phosphate sink, leading to the eventual depletion of ATP, and the corresponding inhibition of tumour growth [21]. Similarly, the fact that cCr-P can act as a store of high-energy phosphate, that is only utilised in times of rapid ATP depletion, may explain the potential beneficial effects of cCr during ischaemia.

4.2. Mitochondrial CK

In vitro studies using MM CK assume that there is only one form of CK that is responsible for phosphorylating creatine and its analogues. Studies have shown that octameric Mt-CK appears to have a central cavity which might be the active site [3]. Perhaps this active site, which is more hidden than the corresponding active site in the cytosolic isoenzymes, has a higher structural specificity and may be a partial explanation for the apparent greater specificity of Mt-CK compared with cytosolic CK.

4.2.1. Creatine-stimulated respiration

The function of Mt-CK is to phosphorylate Cr, with the subsequent release of PCr into the cytosol [5]. This reaction proceeds preferentially in the direction of PCr synthesis. Phosphorylation of Cr by Mt-CK is seen by a stimulation of respiration due to the production of ADP in the intermembrane space [4]. The mechanism for creatine stimulated respiration occurs when Mt-CK is functionally coupled to oxidative phosphorylation. ADP is produced and enters the mitochondrial matrix via the adenine nucleotide translocase (ANT) and stimulates oxidative phosphorylation [5,6,48]. We examined the respiratory responses of the mitochondria, and found that Mt-CK could not use exogenous ATP to phosphorylate GA or β-GPA, whilst it could phosphorylate cCr and creatine. It is not thought that creatine differs from its analogues in terms of its accessibility to Mt-CK, as porin, the transport protein in the outer mitochondrial membrane is permeable to most small molecules. Both Cr and cCr share some common structural features, in that they are both compact molecules, with a similar geometry about the tertiary nitrogen. It was intriguing that GA could not be phosphorylated by Mt-CK, and pointed to the importance of the methyl group in its mechanism of action. As both β-GPA and GA are not phosphorylated by Mt-CK [4,19], it was therefore interesting to establish whether it was solely the lack of the methyl group that was important here, or rather the carbon chain length that also placed some constraint on the specificity of Mt-CK. mGPA was not phosphorylated, indicating that both the methyl group and carbon chain length are important. There appears then to be a greater specificity for Mt-CK compared with cytosolic CK, in terms of both substrate size and geometry.

These results imply a more tightly constrained active site for Mt-CK, compared with cytosolic CK. The kinetic parameters of purified octameric Mt-CK for its substrates are shown in Table 2b [49] and are qualitatively similar to those seen for cytosolic CK. The difference, therefore, in both the structure and specificity of Mt-CK might be related to its coupling to the ANT which effects the kinetics so that Mt-CK phosphorylates Cr using mitochondrially generated ATP, with the ADP generated being taken up preferentially by the ANT [50]. The octameric structure of Mt-CK might be a requirement for its association with the ANT; an indirect result of this may be that the active site of Mt-CK is more specific than its cytosolic counterpart, although of course, Mt-CK and cytosolic CK are different enzymes.

The fact that cCr is utilised by Mt-CK may have benefits in its therapeutic effects in that administering cCr may not lead to the pathologic adaptations that are seen in the feeding of other analogues. As has been discussed, feeding β-GPA feeding leads to the development of paracrystalline occlusions, as well as other energetic modifications, which would be expected to be minimised with cCr feeding.

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