

Phosphorylated guanidinoacetate partly compensates for the lack of phosphocreatine in skeletal muscle of mice lacking guanidinoacetate methyltransferase

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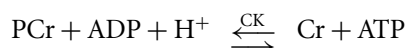
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The effects of creatine (Cr) absence in skeletal muscle caused by a deletion of guanidinoacetate methyltransferase (GAMT) were studied in a knockout mouse model by *in vivo* ³¹P magnetic resonance (MR) spectroscopy. ³¹P MR spectra of hindleg muscle of GAMT-deficient (GAMT^{-/-}) mice showed no phosphocreatine (PCr) signal and instead showed the signal for phosphorylated guanidinoacetate (PGua), the immediate precursor of Cr, which is not normally present. Tissue pH did not differ between wild-type (WT) and GAMT^{-/-} mice, while relative inorganic phosphate (P_i) levels were increased in the latter. During ischaemia, PGua was metabolically active in GAMT^{-/-} mice and decreased at a rate comparable to the decrease of PCr in WT mice. However, the recovery rate of PGua in GAMT^{-/-} mice after ischaemia was reduced compared to PCr in WT mice. Saturation transfer measurements revealed no detectable flux from PGua to γ -ATP, indicating severely reduced enzyme kinetics. Supplementation of Cr resulted in a rapid increase in PCr signal intensity until only this resonance was visible, along with a reduction in relative P_i values. However, the PGua recovery rate after ischaemia did not change. Our results show that despite the absence of Cr, GAMT^{-/-} mice can cope with mild ischaemic stress by using PGua for high energy phosphoryl transfer. The reduced affinity of creatine kinase (CK) for (P)Gua only becomes apparent during recovery from ischaemia. It is argued that absence of Cr causes the higher relative P_i concentration also observed in animals lacking muscle CK, indicating an important role of the CK system in P_i homeostasis.

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To maintain homeostasis and carry out mechanical work, skeletal muscle cells require a constant input of free energy derived from high energy phosphoryl transfer. Hydrolysis of ATP serves as an instantaneous donor of free energy but its concentration in the muscle cell is limited. The phosphorylated form of creatine (Cr), phosphocreatine (PCr), however, is available in much higher concentrations and can regenerate ATP through the transfer of its high energy phosphoryl group to ADP in an equilibrium reaction catalysed by one of the three isoforms of creatine kinase (CK) found in muscle (Wallimann *et al.* 1992; Wyss & Kaddurah-Daouk, 2000):



One of the main functions attributed to the PCr–CK system is that of a temporal energy buffer for high energy phosphates by keeping ATP/ADP ratios balanced

(Wallimann *et al.* 1992). Another function that has been ascribed to the PCr–CK system is that of a spatial energy buffer, or energy shuttle, linking sites of high energy phosphate production (mitochondria) to utilization sites (e.g. myofibrils, sarcoplasmic reticulum) (Bessman & Geiger, 1981). However, the mechanism and importance of this transport in muscle are topics of ongoing debate (e.g. Meyer *et al.* 1986; Wallimann *et al.* 1992; Wyss & Kaddurah-Daouk, 2000; Dzeja & Terzic, 2003).

The importance of intact Cr metabolism in humans was recently highlighted by the identification of a Cr deficiency syndrome caused by a deficiency of guanidinoacetate methyltransferase (GAMT, EC 2.1.1.2) due to mutations in the human GAMT gene, which leads to severe symptoms including mental retardation and muscle hypotonia (Stöckler *et al.* 1994). GAMT is an essential enzyme in the biosynthesis of Cr where it catalyses the final step. The first

step in this biosynthesis, catalysed by L-arginine : glycine amidinotransferase, consists of the transfer of the amidino group from arginine to glycine to yield L-ornithine and guanidinoacetic acid (Gua). Subsequently, the amidino group of Gua is methylated by GAMT to give Cr and, since it is assumed that in vertebrates this process mainly occurs in the pancreas and liver, Cr is exported to the blood and taken up by tissues such as muscle and brain (Wyss & Kaddurah-Daouk, 2000). Since a constant fraction of Cr is converted non-enzymatically to creatinine and excreted daily, biosynthesis and/or dietary Cr sources are needed to maintain a constant body pool of Cr (Walker, 1979).

In mice, studies of the physiological significance of the PCr-CK system have mainly focused on knockout mice lacking one or more of the CK isoforms (e.g. van Deursen *et al.* 1993; Steeghs *et al.* 1997; In 't Zandt *et al.* 1999, 2003) or mice fed creatine analogues (van Deursen *et al.* 1994; Boehm *et al.* 1996; among others). Recently, GAMT-deficient knockout mice (GAMT^{-/-}) have become available (Schmidt *et al.* 2004) that completely lack the essential enzyme GAMT and thus cannot form Cr. This new mouse model provides an excellent opportunity to study the function of the PCr-CK system in energy metabolism from a different perspective.

Magnetic resonance spectroscopy (MRS) allows non-invasive assessment of various compounds central in the study of energy metabolism related to the PCr-CK system (Meyer *et al.* 1982) and is instrumental in the diagnosis of Cr deficiency syndromes in human patients. Our ³¹P and ¹H MRS measurements with GAMT^{-/-} mice validated their use as an animal model for creatine deficiency as they were in agreement with observations in patients (Renema *et al.* 2003). So far, in one patient only, ³¹P MRS of the calf muscle has been performed (Schulze *et al.* 2003) which showed a strongly reduced PCr signal along with a new signal that, in brain, had previously been assigned to phosphorylated Gua (PGua) (Frahm & Hanefeld, 1997). In addition to the measurement of steady-state compound levels using MRS, the method of saturation transfer (ST) (Forsén & Hoffman, 1963) provides a window on enzyme kinetics *in vivo*. During steady-state conditions, ST can be used to measure unidirectional rate constants of chemically exchanging compounds like PCr and ATP (Meyer *et al.* 1982). In ST measurements of the brain of a GAMT-deficient patient, flux between ATP and PGua was decreased below detection level (Frahm & Hanefeld, 1997).

Despite the absence of Cr biosynthesis, GAMT^{-/-} mice are viable and show only minor overt abnormalities (Schmidt *et al.* 2004). Since possible adaptations and subtle deviations that result from this deficiency could elucidate the function of the Cr circuit *in vivo*, the aim of the present study was to investigate the consequences of GAMT deficiency on muscle energy metabolism. We examined muscle energy metabolism of GAMT^{-/-} mice

non-invasively during rest and ischaemia using ³¹P MRS and ST. To study the effects of Cr supplementation on enzyme kinetics, a separate group of GAMT^{-/-} animals was supplemented with Cr and their muscles subjected to ischaemia. By comparing the results of the present investigation to the results from studies on CK-deficient mice, a distinct metabolic phenotype was uncovered for GAMT^{-/-} mice.

Methods

Animals

GAMT^{-/-} mice were generated by homologous recombination in embryonic stem cells (Schmidt *et al.* 2004). Homozygous and heterozygous wild-type (WT) littermates of the GAMT^{-/-} animals were used as a reference (Schmidt *et al.* 2004). Adult animals (> 6 months old) were measured in the present study. Animals were given free access to standard chow based on vegetable protein (ssniff Spezialdiäten GmbH, Soest, the Netherlands, R/M-H) to eliminate possible Cr content.

During the MR experiments, all animals were anaesthetized with 1.5% isoflurane in a gas mixture of 50% O₂ and 50% N₂O delivered through a face mask. Rectal temperature was monitored using a fluoroptic thermometer (Luxtron 712, Santa Clara, CA, USA) and maintained at 37.0 ± 1°C using a warm water bed. Breathing frequency was monitored optically (Sirecust 401, Siemens). All experiments were approved by the animal ethics committee of the University Medical Centre Nijmegen and animals were killed by cervical dislocation after termination of the study.

MR experiments

MRS measurements were carried out on a 7.0 T, 120 mm horizontal bore, magnet (Magnex Scientific, Abingdon, UK) interfaced to a S.M.I.S. spectrometer (Surrey Medical Imaging Systems, Surrey, UK) operating at 121.53 MHz for ³¹P. A three-turn solenoid coil was used for ³¹P MR measurements, in combination with an Alderman-Grant type of proton coil for shimming of the magnetic field.

Ischaemia measurements were carried out on seven WT (3 males, 4 females) and seven GAMT^{-/-} mice (4 males, 3 females). A diaphragm plate which allowed reversible and reproducible obstruction of blood flow through the hind-limb (Heerschap *et al.* 2004) was used to apply ischaemia. To assess basal metabolic levels a ³¹P MR spectrum with a high signal-to-noise ratio (SNR), employing a repetition time (TR) of 7000 ms at 76 averages, was recorded during resting conditions. Subsequently, the ischaemia protocol was started with the recording of ³¹P MR spectra with higher temporal resolution (TR = 1400 ms, 76 averages) for 7 min prior to ischaemia as control, during the 25 min of ischaemia and during 16 min of recovery.

All experiments were carried out with a pulse-acquire sequence using a radio frequency (RF) pulse with a duration of 40 μ s.

To assess the flux from γ -ATP to PCr or PGua, ST measurements were performed on six WT and six GAMT $^{-/-}$ mice (3 males and 3 females in both groups). After obtaining a 31 P MR spectrum during resting conditions (TR = 7000 ms, 128 averages) the γ -ATP signal was selectively saturated for six different durations (500–5000 ms) prior to acquisition (TR = 7000 ms, 64 averages). To correct for any power spill-over of the selective RF pulse, control spectra were recorded with a selective saturation pulse applied at the ‘mirror frequency’ with respect to the PCr or PGua resonance frequencies.

Three groups of GAMT $^{-/-}$ animals were supplemented with 2 g (kg body weight) $^{-1}$ day $^{-1}$ Cr monohydrate (Sigma C0780) dissolved in the drinking water (Ipsiroglu *et al.* 2001). Saccharose (1.6 g (kg body weight) $^{-1}$ day $^{-1}$) was added to the drinking water of the supplemented animals to mask the bitter taste of the Cr monohydrate (Ipsiroglu *et al.* 2001). One group ($n = 4$, 3 females, 1 male) was supplemented for 1 day (24 h), the second group ($n = 6$, 5 females, 1 male) was supplemented for 2 days (48 h) and finally a third group ($n = 4$, 3 females, 1 male) was supplemented for 1 month. In the group that was supplemented for 48 h ischaemia measurements were carried out as described above.

Finally, since a repetition time of 1400 ms results in partly saturated phosphate spin systems, fully relaxed 31 P MR spectra (TR = 25 s, 64 averages) were recorded from six WT and six GAMT $^{-/-}$ animals to obtain saturation correction factors and T1 relaxation times for PCr, PGua and γ -ATP.

Data analysis of MR spectra

Spectra of the 31 P MR measurements were analysed using MRUI software (<http://www.mrui.uab.es/mrui/mruiHomePage.html>) and peak areas were obtained by fitting the signals to Lorentzian line shapes with fixed first-order phase correction.

31 P MR spectra recorded during resting conditions were analysed with no fixed signal dampings except for the 31 P MR spectra recorded after supplementing Cr where the damping of PGua was set equal to the damping of PCr.

For the signals obtained in the ischaemia experiments, the damping of the inorganic phosphate (P_i) signal was set at a fixed ratio of 1.3 times the PCr (WT) or PGua (GAMT $^{-/-}$) peak (empirically determined). Due to a decreased SNR in GAMT $^{-/-}$ animals, the line width of the PGua peak was constrained for each individual mouse as the average line width per stage of the ischaemic protocol: prior to ischaemia, during ischaemia and after ischaemia. For the feeding experiment, the damping of the PGua peak was set equal to that of the PCr peak. All peak areas were

normalized to the average β -ATP signal before ischaemia and corrected by using PCr/ATP, PGua/ATP and P_i /ATP ratios determined from fully relaxed spectra. Correction factors for PGua/ATP and P_i /ATP in GAMT $^{-/-}$ animals were assumed to be equal to PCr/ATP and P_i /ATP ratios in WT animals. The SNR of the β -ATP signal was calculated as the average signal intensity before ischaemia.

The rates of the PCr and PGua signal recovery after ischaemia were analysed by expressing the signal as a percentage of the initial value before ischaemia and fitting this normalized value to a mono-exponential function (Meyer, 1988) by the Levenberg-Marquardt least squares method using Graphpad software (GraphPad Prism, San Diego CA, USA):

$$M(t_{\text{rec}}) = M_0 - M_{\text{cons}} \exp(-t_{\text{rec}}/\tau) \quad (1)$$

where $M(t_{\text{rec}})$ is the amount of signal at t_{rec} seconds after the start of the recovery period, M_0 is the intensity after recovery from ischaemia, M_{cons} is the difference between the PCr or PGua level at the end of ischaemia and M_0 , and τ is the time constant for signal recovery. The initial rate of signal recovery (V_i) was calculated as the first time derivative of this function (e.g. Foley & Meyer, 1993):

$$V_i = M_{\text{cons}}/\tau \quad (2)$$

P_i signal recovery was fitted to a similar function as described in eqn (1). Tissue pH was calculated from the shift in resonance position (S) of the P_i peak compared to the position of PCr (WT). In GAMT $^{-/-}$ animals, where a PCr signal is absent, the resonance position of the PGua signal was determined at 0.44 p.p.m. upfield of PCr. The following equation was used to calculate pH:

$$\text{pH} = 6.75 + \log((3.27 - S)/(S - 5.69)) \quad (3)$$

(Taylor *et al.* 1983) where S is the chemical shift difference between PCr and P_i .

The resting ATPase rate of the hindleg muscle complex was calculated from the slope of a linear regression line through the first four points during the ischaemic period of the PCr/ATP and PGua/ATP plots as pH did not decline during this period (Blei *et al.* 1993; Marcinek *et al.* 2004). As the increase of P_i /ATP during the ischaemic period could also elucidate changes in ATPase activity, the slope of this parameter was calculated in the same way.

In the analysis of the ST experiment, signal intensities of PGua and PCr after irradiation of γ -ATP were normalized to the signal intensity without irradiation. After subtraction of the control spectra, PCr and PGua signal intensities were fitted to a mono-exponential function (Brindle, 1988; Meyer *et al.* 1982) by the Levenberg-Marquardt least squares method using Graphpad software (GraphPad Prism):

$$M_t/M_0 = 1 - k_{\text{for}}(T_{1i}(1 - \exp(-t/T_{1i}))) \quad (4)$$

where, after different saturation times (t), the signal intensity at this time (M_t) is divided by the signal intensity without saturation (M_0), k_{for} is the pseudo-first-order unidirectional rate constant for the CK reaction from PCr or PGua to γ -ATP and T_{1i} is the spin-lattice relaxation time measured in the presence of saturation of γ -ATP. Tissue concentrations of PCr and PGua were calculated with an assumed resting ATP concentration of 7.8 mM (In 't Zandt *et al.* 2003) and the PCr/ATP and PGua/ATP ratios obtained from the spectra with high SNR (TR = 7000 ms, 76 averages) corrected for partial saturation effects.

Chemical determination of ATP levels

Since possible differences in SNR could be due to alterations in ATP levels between mouse types, resting ATP levels of the calf were determined chemically in five WT and six *GAMT*^{-/-} mice. As normalization to total Cr to correct for variations caused by the variable presence of non-muscle elements in the dry tissue powder is not possible in *GAMT*^{-/-} animals due to the absence of Cr, chemically determined ATP levels were only used for the comparison of SNR values.

Animals were anaesthetized with dromicum-hypnorm-H₂O (1 : 1 : 2, 0.01 ml (g body weight)⁻¹). Left and right gastrocnemius, plantaris, soleus (GPS) muscle complexes were exposed and immediately clamp-frozen in brass tongs pre-cooled with liquid nitrogen and stored at -80°C. Muscles frozen in nitrogen were

pulverized in a mortar under constant addition of liquid nitrogen, freeze-dried overnight and stored in liquid nitrogen until further analysis. Metabolites were separated and quantified using a high-performance liquid chromatography (HPLC) system using RP-18 columns (Hewlett Packard) as described previously (Karatzafiri *et al.* 1999). The HPLC system consisted of a Binary LC pump (Model 250, Perkin-Elmer, USA), an auto sampler with cooling tray and automatic injector (Basic Marathon, Spark Holland, the Netherlands) and a variable wavelength ultraviolet (UV) spectrophotometric detector (Model 795A, Applied Biosystems, The Netherlands). The size of the injection loop was 20 μ l and the UV absorption was measured at 254 nm. ATP peaks were identified and quantified by a chromatography data system (model 717; Axxiom Chromatography, CA, USA) by comparing the peak heights of samples with those of external standards (Karatzafiri *et al.* 1999) and expressed in micromole per gram dry weight ($\mu\text{mol (g dry weight)}^{-1}$).

Statistics

Normalized signal intensities of ³¹P MR spectra during rest and ischaemia, SNR and V_i values were compared between *GAMT*^{-/-} and WT mice with a Student's t test. P_i /ATP ratios before and after 1 month of Cr supplementation were compared with a paired t test. The τ values derived from the mono-exponential curves of the recovery after ischaemia and the slopes derived from the linear regression were compared with an F test (GraphPad Prism). Unless stated otherwise, all results were considered significantly different when $P < 0.05$ and are presented as means \pm s.e.m.

Results

Resting conditions

A comparison of ³¹P MR spectra of *GAMT*^{-/-} mice and WT mice under resting conditions revealed a negligible PCr signal for *GAMT*^{-/-} mice along with a new signal at 0.44 p.p.m. which was assigned to PGua, the immediate precursor of Cr (Fig. 1). The resting metabolite level of PGua in *GAMT*^{-/-} mice, determined from ³¹P MR spectra and normalized to the β -ATP peak, did not differ significantly from the PCr/ATP ratio in WT mice (Table 1). Furthermore, resting tissue pH was not significantly different between WT and *GAMT*^{-/-} animals (7.20 ± 0.02 and 7.19 ± 0.04 , respectively). Relative P_i levels, however, were significantly higher in *GAMT*^{-/-} animals (Table 1). The SNR of the β -ATP signal was almost 2-fold lower in *GAMT*^{-/-} animals compared to WT animals (46.0 ± 2.5 and 83.9 ± 2.3 , respectively) ($P < 0.01$). T1 relaxation times were 4.0 ± 0.9 s for PGua and 3.0 ± 0.5 s for PCr and did not differ significantly. The T1 relaxation time of γ -ATP was 1.5 ± 0.3 s.

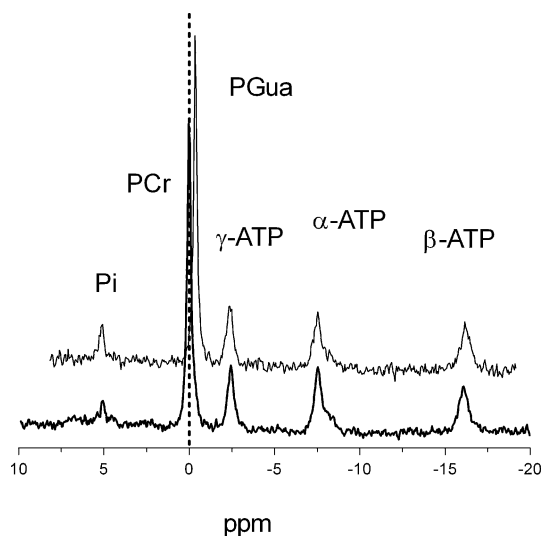


Figure 1. *In vivo* ³¹P MR spectra of the mouse hindleg at rest. Spectra are of a *GAMT*^{-/-} mouse (top) and WT mouse (bottom), TR = 7000 ms, 128 averages. A vertical dashed line is positioned at 0 p.p.m. to show that the large resonance in the top spectrum is indeed PGua at 0.44 p.p.m. upfield from PCr. Peaks for inorganic phosphate (P_i), phosphocreatine (PCr), phosphorylated guanidinoacetate (PGua) and the three resonance positions of ATP (α, β, γ) are visible.

Table 1. High energy phosphate ratios in GAMT^{-/-} and WT hindleg muscle before and after Cr supplementation

	P _i /ATP	PCr/ATP	PGua/ATP
WT	0.40 ± 0.04	3.16 ± 0.10	ud
GAMT ^{-/-}	0.65 ± 0.04§	ud	3.04 ± 0.06
GAMT ^{-/-} (sup 1 day)	0.81 ± 0.06§	1.93 ± 0.11§	2.89 ± 0.20
GAMT ^{-/-} (sup 2 days)	0.60 ± 0.04§	2.18 ± 0.13§	1.91 ± 0.15*
GAMT ^{-/-} (sup 1 month)	0.26 ± 0.03*	2.89 ± 0.03	ud

§ Significantly different from WT animals; * significantly different from GAMT^{-/-} animals before supplementation. Abbreviations: WT, WT animals; GAMT^{-/-}, GAMT^{-/-} animals; GAMT^{-/-} (sup 1 day), GAMT^{-/-} animals supplemented with Cr for 24 h; GAMT^{-/-} (sup 2 days), GAMT^{-/-} animals supplemented with Cr for 48 h; GAMT^{-/-} (sup 1 month), GAMT^{-/-} animals supplemented with Cr for 1 month; PCr, phosphocreatine; P_i, inorganic phosphate; ud, undetectable.

A small but significant difference in the chemically determined ATP concentrations was found between GAMT-deficient and WT mice ($11.1 \pm 1.2 \mu\text{mol (g dry weight)}^{-1}$ and $15.6 \pm 1.1 \mu\text{mol (g dry weight)}^{-1}$, respectively).

Ischaemia

As the PCr-CK system is important under ischaemic conditions when oxidative phosphorylation is shut down, we subjected GAMT^{-/-} animals and WT animals to 25 min of ischaemia and monitored changes in the compounds detectable by ³¹P MRS during ischaemia and recovery thereafter. During ischaemia, changes in normalized signal intensities of PCr and P_i in WT mice and PGua and P_i in GAMT^{-/-} mice were observed (Fig. 2A and B). Interestingly, during the ischaemic period PGua in GAMT^{-/-} animals declined at a rate similar to PCr in WT animals. The resting ATPase rate, determined from the slope of the decline of PCr and PGua, did not differ significantly between WT and GAMT^{-/-} mice (Table 2). A similar calculation from the slopes of the increase in P_i/ATP values in both mouse types also showed no significant difference. In GAMT^{-/-} mice, P_i levels were significantly elevated prior to ischaemia and remained significantly higher during and after ischaemia. Relative muscle ATP levels of both mouse types remained constant throughout the ischaemic period (Fig. 2C). Tissue pH did not differ between mouse types at the start of the ischaemic period, while pH was slightly lower in GAMT^{-/-} mice towards the end of the ischaemic period (6.97 ± 0.01 for WT and 6.85 ± 0.03 for GAMT^{-/-} mice) (Fig. 3).

It has been stated that measurements of PCr recovery after depletion can be used as an index of relative oxidative capacity or mitochondrial content in muscle (e.g. Kemp *et al.* 1993). To assess these recovery kinetics, a mono-exponential function was fitted to the recovery of PCr, PGua and P_i and the resulting τ values revealed interesting differences (Table 2). The recovery of the PGua signal in GAMT^{-/-} animals was significantly slower than the recovery of PCr in WT animals ($P < 0.01$), with τ

values of PCr and PGua of 107 ± 14 and 383 ± 83 s, respectively. In contrast to the recovery of PGua, τ values of P_i/ATP recovery did not differ significantly between mouse types. By calculating V_i from the time constants, flux rates from γ -ATP to PCr and PGua can be compared. V_i values for PCr in WT and PGua in GAMT^{-/-} animals were 0.117 ± 0.01 and $0.034 \pm 0.003 \text{ mm s}^{-1}$, respectively, and differed significantly ($P < 0.01$) (Table 2).

Saturation transfer

As the difference in recovery rate after ischaemia could be due to a difference in enzyme kinetics, ST experiments were performed (Fig. 4). With increasing irradiation times, no decrease in signal intensity of PGua was observed (Fig. 5). The k_{for} value calculated for PCr was $0.39 \pm 0.03 \text{ s}^{-1}$, resulting in a forward flux through the CK reaction of $9.7 \pm 0.5 \text{ mm s}^{-1}$ (Table 2). The absence of a detectable decrease in the PGua signal with increasing irradiation times shows that no chemical exchange could be detected in this period. As was shown previously for mice lacking the cytosolic form of CK (van Deursen *et al.* 1993), the absence of a detectable decrease in a signal in this type of ST experiment indicates at least a 20-fold reduction in reaction rates compared to CK in WT mice.

Cr supplementation

To observe the effects of Cr supplementation, $2 \text{ g (kg body weight)}^{-1} \text{ day}^{-1}$ Cr was added to the diet of GAMT^{-/-} animals for three different time periods and this resulted in the appearance of a PCr signal in the ³¹P MR spectrum (Fig. 6). With increasing Cr supplementation durations, the ratio of PCr/PGua increased from 0.63 ± 0.08 to 1.19 ± 0.13 after 24 and 48 h, respectively, and differed significantly. Cr supplementation for 1 month resulted in a complete disappearance of the PGua peak (Fig. 6), a significant reduction in the P_i/ATP ratio compared to the pre-supplementation value and PCr/ATP levels comparable to values found in WT mice (Table 1).

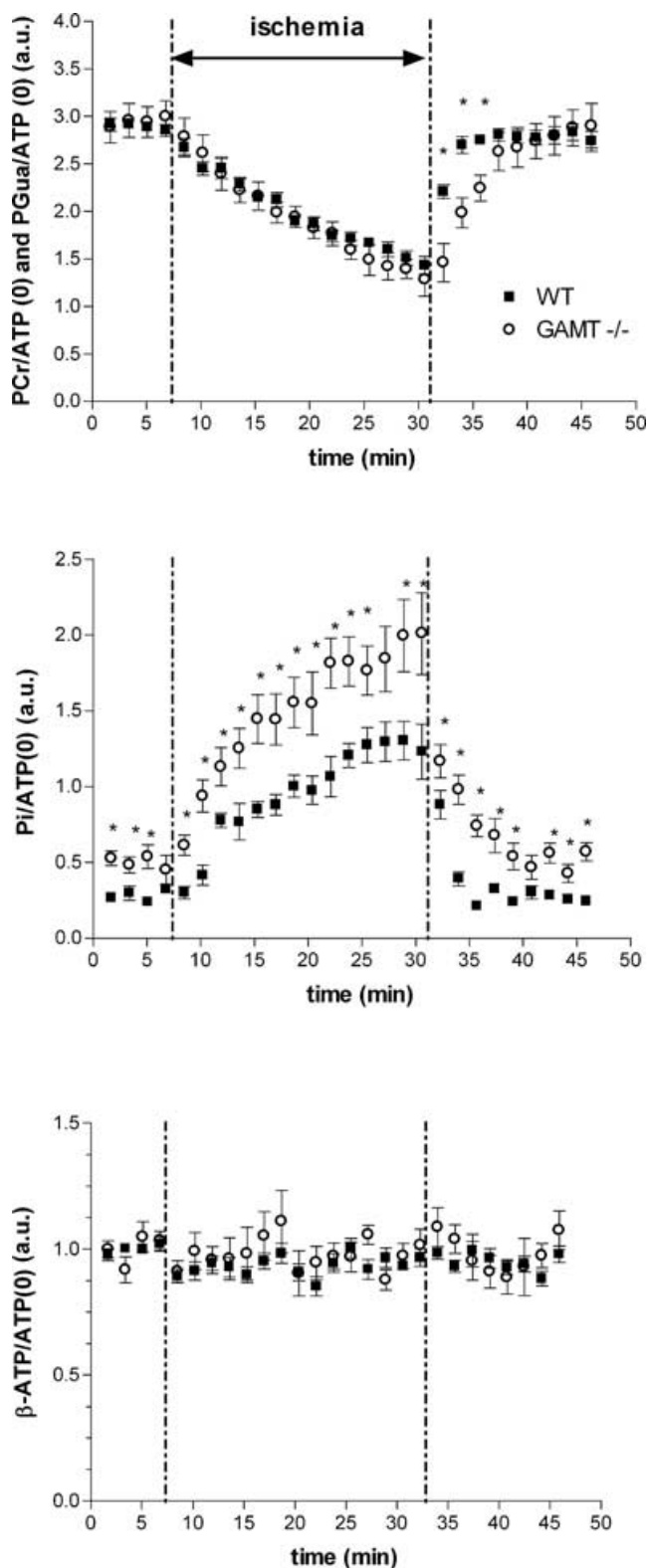


Figure 2. Time plots of signal intensities normalized to the β -ATP signal before, during and after ischaemia. Time courses of changes in PCr (WT) and PGua (GAMT^{-/-}) (A), P_i (B) and ATP (C) are shown as means \pm s.e.m. for WT ($n = 7$, ■) and GAMT^{-/-} ($n = 7$, ○). * Significantly different from WT at $P < 0.05$. a.u., arbitrary units.

To assess whether Cr feeding had an effect on the recovery rate after ischaemia, the group of animals that was supplemented with Cr for 48 h was subjected to the same ischaemia protocol as the WT and non-supplemented GAMT^{-/-} mice. After feeding of Cr, the τ and V_i values derived from changes in the PGua signal did not differ significantly from those of non-supplemented GAMT^{-/-} mice. For the recovery of PCr, however, τ and V_i values were significantly increased in the supplemented GAMT^{-/-} animals compared to WT animals (Table 2).

Discussion

This study demonstrates that despite inhibition of the final step in Cr biosynthesis, as shown by the absence of a PCr peak in the ³¹P MR spectrum, GAMT-deficient animals can cope with ischaemic stress. By using Gua, the immediate precursor of Cr, and thus PGua for high energy phosphoryl transfer they appear to compensate for the lack of Cr and PCr. Compared to WT animals they showed a higher relative P_i signal intensity, a slower recovery of PGua than of PCr after ischaemia and clearly reduced enzyme kinetics. Cr supplementation caused a rapid appearance of the PCr signal and a reduction of the P_i /ATP ratio to WT levels but did not alter PGua recovery kinetics after ischaemia.

Resting conditions

PCr is virtually absent in skeletal muscle of GAMT^{-/-} mice. In a previous study by our group on these mice (Renema *et al.* 2003), the ³¹P MR spectra of skeletal muscle still showed a signal for PCr which was probably caused by trace amounts of Cr in the food. Recent observations have shown that the mice practice coprophagia, which could also account for residual PCr if GAMT^{-/-} mice are housed with mixed-genotype groups. Consequently, mice used for the experiments reported here were housed according to genotype and fed Cr-free food. Our earlier hypothesis that residual PCr signals could result from oral intake is confirmed by the observation that after controlled Cr supplementation, a PCr signal appeared in GAMT^{-/-} muscle. This PCr peak was visible after only 24 h of supplementation, despite the fact that Gua partially inhibits Cr uptake in skeletal muscle (Fitch & Chevli, 1980; Fitch *et al.* 1968). Longer supplementation of GAMT^{-/-} mice with Cr caused a progressive decrease in the PGua signal intensity as the PCr signal increased. Since Gua uptake in skeletal muscle occurs through the Cr transporter (Wyss & Kaddurah-Daouk, 2000), the decrease in PGua is most likely caused by a competitive inhibition of Gua uptake by Cr (Fitch *et al.* 1968).

After 1 month of Cr supplementation in GAMT^{-/-} mice, no PGua peak was visible and PCr/ATP levels

Table 2. Recovery kinetics and flux parameters of WT and GAMT^{-/-} hindleg muscle

	WT	GAMT ^{-/-}	GAMT ^{-/-} (sup 2 days)
τ_{PCr} (s)	107 ± 14	n.a.	31 ± 40§
τ_{PGua} (s)	n.a.	383 ± 83**	294 ± 131*
τ_{P_i} (s)	145 ± 29	168 ± 35	n.d.
$V_{i\text{PCr}}$ (mm s ⁻¹)	0.117 ± 0.01	n.a.	0.38 ± 0.05§§
$V_{i\text{PGua}}$ (mm s ⁻¹)	n.a.	0.034 ± 0.003**	0.031 ± 0.003**
Forward flux (mm s ⁻¹)	9.7 ± 0.5	< 0.5†	n.d.
ATPase rate (mm s ⁻¹)	0.027 ± 0.003	0.043 ± 0.006	n.d.

§ $P < 0.05$; §§ $P < 0.01$: significantly different from WT animals; * $P < 0.05$, ** $P < 0.01$: significantly different from PCr values in WT animals. † Calculated upper value. Abbreviations: n.a. = not applicable; n.d. = not determined; WT, WT animals; GAMT^{-/-}, GAMT^{-/-} animals; GAMT^{-/-} (sup 2 days), GAMT^{-/-} animals supplemented with Cr for 48 h; τ , time constants of recovery after ischaemia of phosphocreatine (PCr), phosphorylated guanidinoacetate (PGua) and inorganic phosphate (P_i); V_i , initial rates of recovery.

were comparable to WT animals. Furthermore, these animals showed a significant reduction in P_i/ATP levels compared to the level before supplementation. Interestingly, the P_i/ATP levels in these animals were similar to WT littermates, indicating a change to the WT muscle phenotype.

The observed effect of Cr on the P_i/ATP ratio could explain the absence of a difference in P_i/ATP levels between WT and GAMT^{-/-} mice where (P)Cr was present continuously (Renema *et al.* 2003). However, as only the mice supplemented with Cr for 1 month showed a significant decrease in their relative P_i content, this reduction apparently needs some time to take effect.

The almost 2-fold lower SNR of the β -ATP peak in GAMT^{-/-} muscles could result from a number of factors including a lower ATP level and decreased muscle mass. As the chemically determined ATP values of GAMT^{-/-} hindleg muscle only differed slightly from WT muscle, it is unlikely that a decrease in resting ATP level alone causes the decreased SNR. Recently we observed (Kan *et al.* 2004) that GAMT^{-/-} medial gastrocnemius muscle weight was significantly lower than in WT mice, indicating that a reduced muscle mass contributes to this decreased SNR.

Comparison to creatine kinase knockout mice

PCr and PGua. Since it has been shown that the cytosolic isoform of CK phosphorylates Cr analogues (e.g. Fitch & Chevli, 1980; van Deursen *et al.* 1994; Boehm *et al.* 1996), it is likely that CK activity is responsible for the phosphorylation of Gua. Therefore, it is interesting to compare the present results with those from studies on mice lacking muscle CK.

The constant ATP levels throughout the entire ischaemic period indicate that, despite the absence of Cr, GAMT^{-/-} animals can sustain the stress of short-term ischaemia well. A constant ATP concentration

in skeletal muscle during ischaemia has also been found in mice lacking the mitochondrial isoform of CK (ScCKmit^{-/-}) and in mice lacking the cytosolic isoform of CK (M-CK^{-/-}) but not in mice lacking both isoforms (M-CK/ScCKmit^{-/-}) (In 't Zandt *et al.* 1999). This indicates that the absence of Cr due to GAMT deficiency does not compromise ischaemic skeletal muscle energy metabolism to the same extent as a complete absence of CK. The rate of the decrease of PGua during ischaemia shows that the compound is metabolically active and that GAMT^{-/-} mice can use Gua for high energy phosphoryl transfer at a similar rate as PCr is used by WT animals. Therefore, despite the severe reduction in forward flux as indicated in the ST experiments, the reaction rate of PGua is still sufficient to meet energy demand during ischaemia as ATP levels remained constant. Apparently the resting ATPase rate, determined by the decrease of PCr and PGua at constant pH, can still be matched by the flux of PGua to γ -ATP (Table 2). This was also shown in M-CK^{-/-} animals, where despite a considerably lower flux rate (van Deursen *et al.* 1993), PCr levels decreased at a similar rate as in WT animals (In 't Zandt *et al.* 1999).

P_i and pH. During rest and ischaemia relative P_i values and kinetics differ between GAMT^{-/-} mice and CK knockout mice. The higher resting relative P_i concentration found in GAMT^{-/-} mice was absent in both ScCKmit^{-/-} and M-CK^{-/-} mice (In 't Zandt *et al.* 1999), while M-CK/ScCKmit^{-/-} animals displayed a 2- to 3-fold higher P_i/ATP ratio compared to WT mice (Steeghs *et al.* 1998). However, in contrast to GAMT^{-/-} mice, the increase in P_i during ischaemia is severely compromised in the M-CK/ScCKmit^{-/-} mice (In 't Zandt *et al.* 1999).

Since, among other energy signalling molecules (Bose *et al.* 2003), Cr is thought to play an important role in stimulating mitochondrial respiration in concert with the mitochondrial isoform of CK (e.g. Boehm *et al.* 1998; Wallimann *et al.* 1998; Kay *et al.* 2000), the absence

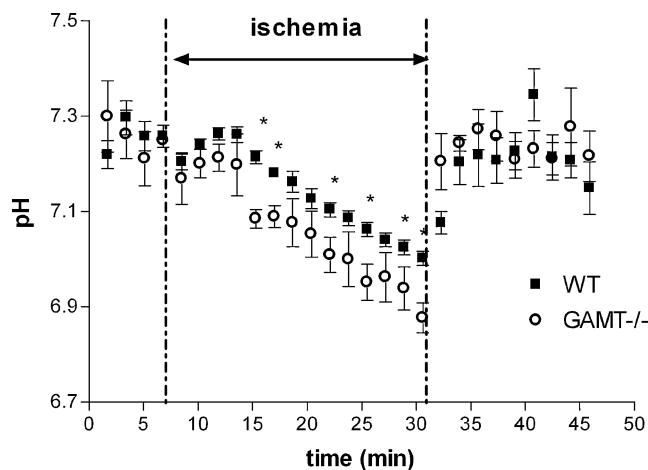


Figure 3. Time curves of the pH changes before, during and after ischaemia

Tissue pH in hindleg muscle of WT ($n = 7$, ■) and $GAMT^{-/-}$ ($n = 7$, ○) mice. Data are shown as means \pm s.e.m. * Significantly different from WT at $P < 0.05$.

of Cr might be crucial here. As ScCKmit is unable to phosphorylate Gua (Boehm *et al.* 1996), Gua cannot substitute for Cr in stimulating mitochondrial respiration in $GAMT^{-/-}$ animals. M-CK/ScCKmit $^{-/-}$ mice also lack

Cr stimulated respiration due to the absence of ScCKmit and M-CK, and the function of ScCKmit cannot be taken over by M-CK as in ScCKmit $^{-/-}$ animals (Boehm *et al.* 1998). Since both mutants display elevated P_i concentrations, it is conceivable that lack of Cr or the inability to phosphorylate this compound can lead to compensatory mechanisms resulting in a shift in P_i homeostasis. This is supported by the fact that in the $GAMT^{-/-}$ animals where (P)Cr was present continuously, no higher resting P_i concentrations were found (Renema *et al.* 2003). In contrast to M-CK/ScCKmit $^{-/-}$ animals, in $GAMT^{-/-}$ animals high energy phosphate transfer from PGua to ATP is still possible through M-CK, thereby producing a higher P_i level during ischaemia.

At the beginning of the ischaemic period, tissue pH remained fairly constant in the WT mice. This is in agreement with other studies on ischaemia (In 't Zandt *et al.* 1999; Marcinek *et al.* 2004) and studies on exercise (e.g. Adams *et al.* 1990; Houtman *et al.* 2001; Crowther *et al.* 2002) and is attributed to proton consumption by PCr hydrolysis. No differences were observed between $GAMT^{-/-}$ and WT mice at the first time points during ischaemia, suggesting that PGua supports proton buffering as well. Similar tissue pH values at the beginning of

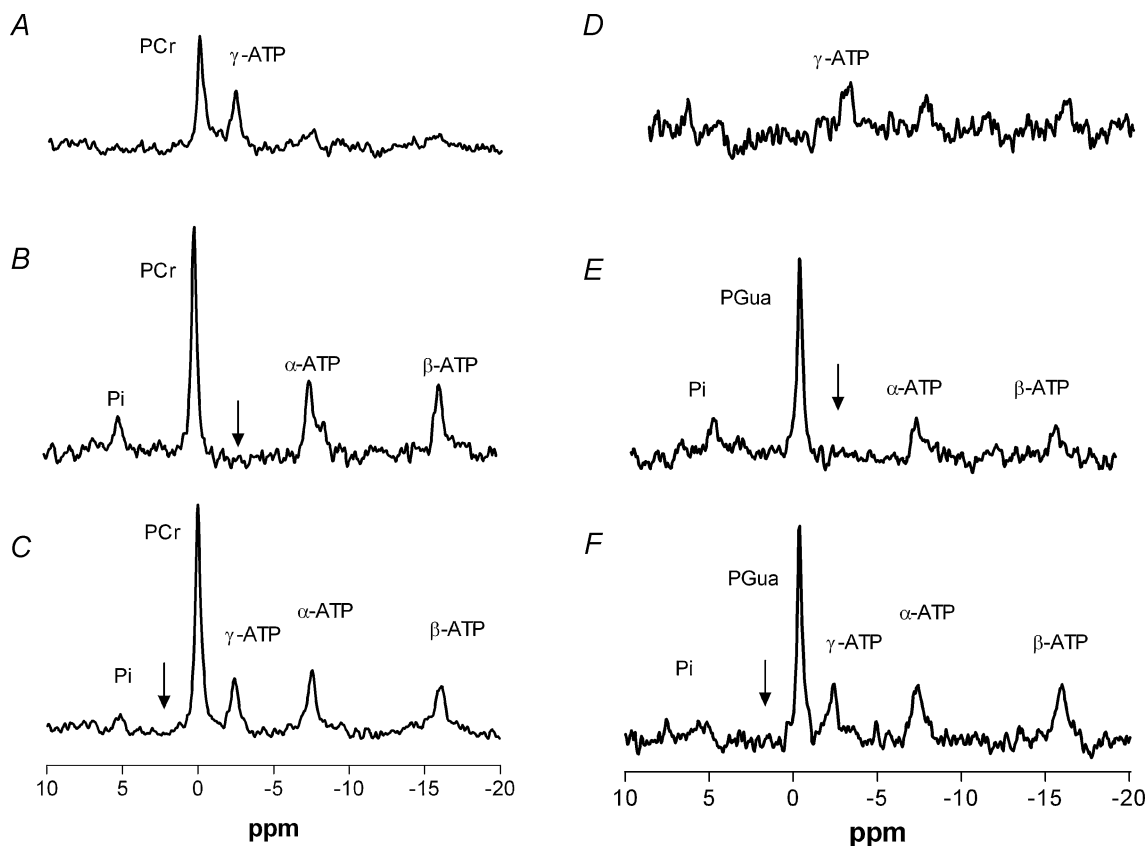


Figure 4. Typical saturation transfer spectra of a WT and a $GAMT^{-/-}$ mouse hindleg

The appropriate control spectra (C and F), a spectrum with a 5000 ms (B) or a 3000 ms (E) saturation pulse applied at the frequency of γ -ATP and the corresponding difference spectra (A and D). Arrows denote the frequency of the selective saturation pulse. Spectra A–C are of a WT mouse, D–F of a $GAMT^{-/-}$ mouse.

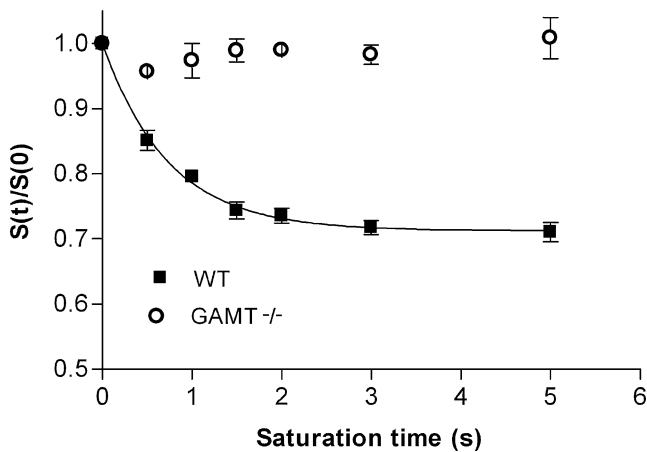


Figure 5. Normalized signal intensities of PCr and PGua with increasing saturation times

Signal intensities of PCr (WT; $n = 6$, ■) and PGua (GAMT^{-/-}; $n = 6$, ○) were normalized to the corresponding signal intensity in the absence of a saturation pulse. Data are shown as means \pm S.E.M.

the ischaemic period were also found in M-CK^{-/-} and ScCKmit^{-/-} mice but not in M-CK/ScCKmit^{-/-} mice where tissue pH immediately started to decline at the onset of ischaemia due to proton production, e.g. by ATP hydrolysis (In 't Zandt *et al.* 1999).

After about 10 min of the ischaemic period a small but significant pH difference is observed between GAMT^{-/-} and WT mice. Since in ischaemic conditions pH will fall due to an increased proton production by glycolysis compared to proton consumption by PCr breakdown (Kemp *et al.* 2001), the earlier decrease in pH observed in GAMT^{-/-} mice could be ascribed to an earlier start of glycolysis caused by higher levels of P_i in GAMT^{-/-} animals (Crowther *et al.* 2002).

Recovery rate after ischaemia and enzyme kinetics

Ischaemia is a relatively slow process and it has been shown that during brief periods of ischaemia, the ATPase rate does not differ from aerobic conditions (Conley *et al.* 1998; Kemper *et al.* 2001). Recovery after ischaemia, however, is a faster process that uncovered some

interesting differences between GAMT^{-/-} and WT mice have been uncovered.

As mentioned above, measurements of PCr recovery after depletion can be used as an index of relative oxidative capacity or mitochondrial content in muscle. After ischaemia, oxygen is available again and recovery of high energy phosphates will be mainly due to ATP production in the mitochondria via oxidative phosphorylation. Since the recovery of PGua in GAMT^{-/-} mice was significantly slower than PCr recovery in WT mice, this delay could be explained by a reduced oxidative capacity. However, since no reduction was found in citrate synthase activity of GAMT^{-/-} mice compared to WT mice (Schmidt *et al.* 2004), it is unlikely that a reduced oxidative capacity caused the delayed recovery of PGua in GAMT^{-/-} mice.

Another factor that could explain the reduced recovery rate of PGua after ischaemia is the absence of Cr-stimulated respiration. In WT animals, Cr increases and PCr decreases during ischaemia, which leads to an increased stimulation of mitochondrial respiration (Walsh *et al.* 2001) as soon as oxygen is available again. In GAMT^{-/-} animals (P)Cr is absent and, as mentioned above, Gua cannot take over the proposed role of Cr since ScCKmit is unable to phosphorylate the compound. This could lead to a less effective stimulation of mitochondrial respiration and thereby a slowing of the PGua recovery rate.

A third potential factor that might influence the recovery rate is the affinity of CK for Gua. Since Gua is 100-fold less reactive with CK compared to Cr *in vitro* (Boehm *et al.* 1996), and as the ST experiments showed a severe reduction of flux through the CK reaction *in vivo*, enzyme kinetics could be reduced to such an extent that they become rate limiting. Furthermore, the initial rate of recovery of PGua (V_i) (Table 2) indicates that the actual flux rate is well below the detection limit of the ST experiment.

Finally, tissue pH has also been shown to influence recovery kinetics (Iotti *et al.* 1993, 2004; Paganini *et al.* 1997). However, in the GAMT^{-/-} animals that were supplemented with 2 g kg⁻¹ Cr for 2 days, tissue pH was intrinsically the same for PCr and PGua recovery, since

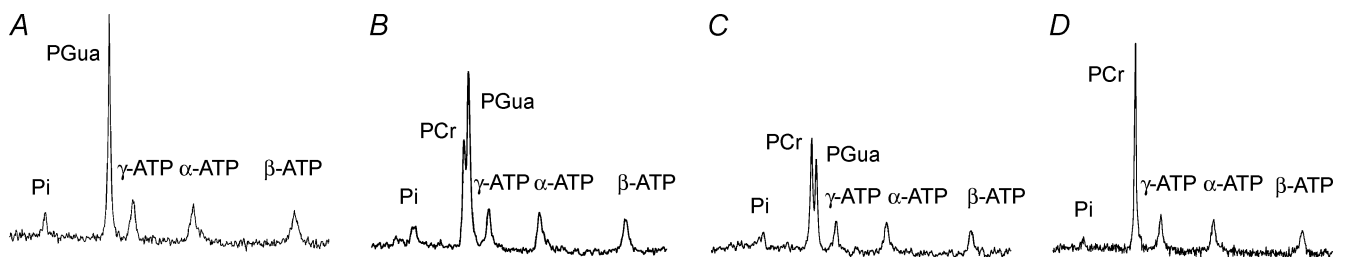


Figure 6. Typical *in vivo* ³¹P MR spectra at rest of the hindleg of four GAMT^{-/-} mice at different stages of Cr supplementation

A, without supplementation. B, after 24 h of Cr supplementation. C, after 48 h of Cr supplementation. D, after 1 month of Cr supplementation. Peaks for inorganic phosphate (P_i), phosphocreatine (PCr), phosphorylated guanidinoacetate (PGua) and the three resonance positions of ATP (α, β, γ) are visible.

they are in the same compartment. Therefore, since the difference in recovery rate of PCr and PGua persisted, it is unlikely that pH alone accounts for the difference.

If the absence of the stimulatory effect of Cr was the major factor influencing the recovery of PGua, we would have expected the recovery rate of PGua to increase after Cr supplementation. However, the recovery of the PGua signal after ischaemia was not significantly elevated in the Cr-supplemented GAMT^{-/-} animals compared to the non-fed GAMT^{-/-} group. The recovery of the PCr signal in the Cr-supplemented GAMT^{-/-} mice was significantly faster than in WT animals. Apparently, Cr is still able to stimulate mitochondrial respiration in GAMT^{-/-} animals without leading to an elevation in the recovery rate of PGua. This strongly suggests that the lowered affinity of CK for Gua limits the recovery of PGua after ischaemia.

In contrast to the significant difference in τ values for the recovery of PGua and PCr, no such difference was found between τ values for P_i of WT and GAMT^{-/-} mice. This could be explained by the fact that P_i has more than one fate after the ischaemic period, whereas PCr and PGua can only be generated from ATP through the CK reaction. Furthermore, at pH values below 6.95, P_i recovery is faster than at higher pH values, possibly due to an increased pH gradient over the mitochondrial membrane (Iotti *et al.* 1993). Since in GAMT^{-/-} animals the pH value at the end of the ischaemic period is below this value, an effect of pH cannot be excluded.

In summary, our results indicate that GAMT^{-/-} mice can use the immediate precursor of Cr Gua for high energy phosphoryl transfer despite the fact that the corresponding enzyme kinetics are severely reduced. These reduced enzyme kinetics cause a significant delay in the recovery of the PGua signal after ischaemia compared to the PCr recovery in WT mice. The effect of Cr supplementation on relative P_i levels in GAMT^{-/-} mice reveals that the absence of Cr or PCr results in a shift in skeletal muscle P_i values. Future experiments involving a more severe metabolic challenge, such as exercise, will provide further details on the use of PGua as an energy donor and the consequences of GAMT deficiency.

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