In vivo brain phosphocreatine and ATP regulation in mice fed a creatine analog

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Holtzman, D., R. Meyers, E. O'Gorman, I. Khait, T. Wallimann, E. Allred, and F. Jensen. In vivo brain phosphocreatine and ATP regulation in mice fed a creatine analog. *Am. J. Physiol.* 272 (*Cell Physiol.* 41): C1567–C1577, 1997.— Mitochondrial and cytosolic creatine kinase (CK) isozymes are active in cells with high and variable ATP metabolic rates. β-Guanidinopropionic acid (GPA), a competitive inhibitor of creatine transport, was used to study the hypothesis that the creatine-CK-phosphocreatine (PCr) system is important in regulating brain ATP metabolism. The CK-catalyzed reaction rate and reactant concentrations were measured in vivo with ^{31}P nuclear magnetic resonance spectroscopy during energy deficit (hypoxia) or high-energy turnover (seizures) states in urethane-anesthetized mice fed GPA, creatine, or standard chow (controls). Brain phosphagen (i.e., cellular energy reserves) or PCr plus phosphorylated GPA (GPAP) concentrations were equal. The phosphagen-to-NTP ratio was lower than in controls. In vivo CK reaction rate decreased fourfold, whereas ex vivo CK activity that was biochemically measured was doubled. During seizures, CK-catalyzed fluxes increased only in GPA-fed mice. Phosphagen increased in GPA-fed mice, whereas PCr decreased in controls. Survival was higher and brain phosphagen and ATP losses were less for hypoxic GPA-fed mice than for controls. In contrast to mice fed GPA, hypoxic survival and CK reactant concentrations during hypoxia and seizures were the same in creatine-fed mice and controls. Thus GPA, GPAP, or adaptive changes in ATP metabolism stabilize brain ATP and enhance survival during hypoxia in mice.

creatine kinase; hypoxia; ^{31}P nuclear magnetic resonance; seizures

IN SKELETAL MUSCLE AND heart, the creatine kinase (CK; EC 2.7.3.2)-catalyzed reaction

$$PCr^- + MgADP^- + H^+ \leftrightarrow Cr + MgATP^+ \quad (1)$$

is thought to be important in the synthesis of cytosolic ATP, the temporal buffering of cellular ATP concentration, and/or the regulation of reactant concentrations, including ATP, ADP, creatine, phosphocreatine (PCr), and pH (3, 27, 29, 32, 52, 56, 59). Like other tissues with large and rapidly changing ATP requirements, brain contains a relatively high PCr concentration and both cytosolic CK (B-CK) and mitochondrial CK (Mi-CK) isoenzymes (26, 27, 38). The brain CK isoenzymes differ from those in skeletal muscle (24, 54). The isoenzymes are not uniformly codistributed in cerebellar and cerebral hemispheres, with B-CK present in both gray and white matter and Mi-CK present predominantly in gray matter, a region of high and variable rates of aerobic glycolysis (23, 28). The hypothesis for the present study is that, in brain as in muscle, the CK-catalyzed reaction and/or reactants are important in regulating ATP with a physiology that reflects the cerebral metabolic heterogeneity.

The physiology of brain ATP metabolism, including the creatine-CK-PCr system, has been studied in vivo with nuclear magnetic resonance (NMR) spectroscopy under conditions of high-ATP turnover (seizures) and energy deprivation (hypoxia, ischemia, or hypoglycemia) (19, 37, 38, 41, 49, 51, 60). During seizures, cerebral hemisphere PCr decreases 20–40% and ATP decreases ~10% while blood flow and aerobic glycolysis double (19, 25, 37, 46, 60). The CK-catalyzed flux increases (41). During mild hypoxia, brain ATP is maintained in association with increased blood flow, O_2 and glucose extraction from blood, mitochondrial electron transport chain oxidation, CK-catalyzed reaction rates, and pH (4, 22, 25, 46, 51). Electrocortical activity slows. More severe or prolonged hypoxia results in reduction of cellular cytochrome aa_3, simultaneous decreases in pH and in PCr and ATP, and a decrease in CK-catalyzed fluxes (22, 49, 51).

The importance of creatine in ATP metabolism, although not its specific function(s), is suggested by extensive changes in ATP metabolic pathways in creatine-depleted skeletal and cardiac muscle. The creatine analog, β-guanidinopropionic acid (GPA), almost completely replaces creatine in muscle when fed to rats or mice (10, 43, 52). GPA, which competitively blocks creatine transport, is phosphorylated by the CK-catalyzed reaction, but both GPA and phosphorylated GPA (GPAP) are poor substrates compared with creatine (7, 11). GPAP decreases with contractions and anoxia in skeletal muscle from GPA-fed mice, suggesting that it can act as substrate for the CK-catalyzed reaction in vivo (10). These in vivo results also indicate that GPA is intracellular. In GPA-fed rats, muscle aerobic glycolytic enzymes and adenylate kinase increase, hexokinase and phosphofructokinase decrease, and concentrations of glucose-6-phosphate and ATP decrease (10, 33, 35, 42). Total CK activity decreases, whereas Mi-CK and the adenine nucleotide translocase increase three- to fourfold (35, 42). These changes in ATP metabolism are associated with decreased fast and intermediate myosin isoenzymes and increased slow forms in fast- and slow-twitch skeletal muscle (34). With these metabolic adaptations, prolonged stimulation produces similar contractile force in skeletal muscles from GPA-fed and control rats (32, 44, 45).

Guanidino compounds, including creatine, PCr, and arginine, are important in regulation of brain energy metabolism. In anoxic hippocampal slices, creatine but not ATP markedly prolongs synaptic transmission and

cell survival (5, 58). Arginine, the substrate for nitric oxide synthase, is thought to be active in regulating cerebral blood flow (16, 48). In contrast to muscle, brain PCr is only partially replaced by GPAP (18). The CK-catalyzed reaction rate with PCr as substrate, measured in vivo by NMR, decreases 60–70%. In further contrast to skeletal muscle, brains from GPA-fed rats show increased total CK with only a small increase in Mi-CK (35). Both tissues show increased adenylate kinase and succinate dehydrogenase. Regulation of ATP has not been studied in brains of GPA-fed animals.

In this study, changes in brain CK-catalyzed reaction rates and reactant concentrations were measured in vivo with ^{31}P-NMR spectroscopy during hypoxia and seizures in mice fed GPA or creatine. Control mice for each group were fed standard laboratory chow. Total CK activity was measured in vitro in brains from the GPA-fed and control mice. The GPA-fed mice showed markedly decreased brain CK-catalyzed reaction rates in vivo, increased total CK activity, enhanced hypoxic survival, and stabilization of ATP during both hypoxia and seizures. In contrast, results in creatine-fed animals were no different from controls.

METHODS

Animals. Female CD-1 albino mice (Charles River Breeding Laboratory, Wilmington, MA) were acquired at 30 days of age and kept in cages of five animals each. After 2 days, ad libitum feedings of standard laboratory chow (controls), chow containing GPA (at 2% by weight), or chow containing creatine (at 2% by weight) (Bioserve, Frenchtown, NJ) were started. For the NMR experiments, mice were fed the respective diets for at least 3 wk. For the analytic measures of CK activity, mice were fed for 6 or 12 wk. The brain phosphagens are the same after all these durations of GPA feedings, since brain GPAP-to-PCr and total phosphagen-to-NTP ratios do not change after 2 wk of feedings (18). Control animals were paired to each group of experimental GPA- or creatine-fed mice throughout the feeding periods and when studied.

Seizures. Mice were anesthetized with urethan (1.875 mg/g). After placement of an intraperitoneal catheter filled with pentylenetetrazole (PTZ) in water (10 mg/ml), the mouse was taped in the NMR probe. Baseline spectra with 2-s (partially saturated) and 10-s (fully relaxed) interpulse intervals (RD) were acquired in room air. A saturation transfer (ST) experiment, which measures the phosphoryl flux between PCr and ATP, was then run. After injection of PTZ (80 mg/g), partially saturated spectra were acquired every 2 min. The ST experiment was repeated after 12 min when spectral changes were stable.

The electroencephalogram (EEG) was acquired in three additional GPA-fed and control mice. Each mouse was anesthetized before biparietal scalp electrodes were placed and the animal was fixed in the NMR probe. The EEG leads were connected to low-pass filters (80 dB at 1 MHz) with shielded cables. The EEGs were recorded for at least 15 min after the PTZ injections. The EEGs were examined for onset and duration of seizure activity by an observer (F. Jensen) who did not know the feeding group. After each experiment, the anesthetized mouse was killed with intraperitoneal pentobarbital (200 mg/kg).

Hypoxia. In preliminary experiments, 8–10 mice in each experimental group and controls were exposed to hypoxic gas mixtures (6, 4, or 2% O_2) for 10 min. The mice were anesthetized with urethane for placement of biparietal scalp electrodes. After recovery from anesthesia, mice were placed in a Lucite chamber maintained at 34°C. The mice were observed, and EEGs and heart rate were recorded in room air, in the hypoxic gas, and again in air.

For NMR studies, mice were anesthetized with intraperitoneal urethane before placement in the probe. Baseline spectra and the ST experiment were acquired. The hypoxic gas mixture then completely replaced room air in the probe within 15 s through a funnel in front of the animal's face. The GPA-fed mice and controls were exposed for 7–8 min to one of the three hypoxic gas mixtures. The creatine-fed and control mice were exposed to either 6 or 4% O_2. Partially saturated spectra were obtained every 2 min. Room air replaced the hypoxic gas after 7 min, and spectra were acquired for 12 min. A second ST experiment was then performed. Body temperatures, monitored by a rectal thermistor, were 33–34°C throughout the experiments.

NMR experiments. Spectra were obtained in the Fourier transform mode at 145.587 MHz with a custom-built spectrometer and an Oxford 8.9-cm vertical bore superconducting magnet (8.45 T). Spectra were localized to brain by means of an 8-mm-diameter Teflon-covered copper surface coil. The surface coil was matched and tuned to the ^{31}P-NMR frequency after the animal was placed in position with the coil centered on the scalp behind the supraorbital ridges. Field homogeneity was optimized to a line width of <0.2 parts/million, using the brain water signal. Spectral acquisition time was 73 ms after an excitation pulse of 12 μs. The spectral width was 14,000 Hz with 1,024 time domain points collected and zero filled to 4,096. The sum of 56 time domain signals (free induction decays) was multiplied by an exponential filter with 25-Hz line broadening before Fourier transforming and baseline deconvolving (1). Spectra were processed with NMR I software (New Methods Research, East Syracuse, NY).

Changes in reactant concentrations were measured by integrating spectral peaks. Values from initial 2-s RD spectra were compared with spectra acquired during and after hypoxia or during seizures. Because of the overlap of the PCr and GPAP peaks, the area under the two peaks was integrated as a measure of total phosphagen concentration. Changes in ATP concentration were estimated from the β-NTP peak, to which there are no contributions from NAD or ADP (14). This estimation assumes that only ATP changes acutely during seizures and hypoxia and that brain ATP is ~67% of the total NTP in all mice (6, 30, 57). Brain pH was calculated from the chemical shift of P_i relative to PCr (36).

As shown in Fig. 1, the rate of phosphorus transfer from PCr to ATP was measured by using the ST experiment (2, 12, 17). Selective saturation of the signal from the ATP γ-phosphorus nucleus was achieved with a 3.5-s low-power saturating pulse centered on the γ-NTP frequency. The usual excitation pulse was then applied, and the free induction decay was acquired to record the net z-magnetization. The spectra shown were the sum of 64 acquisitions with 3.5-s RD. A second spectrum was acquired after placing the saturating pulse frequency an equal distance from PCr on the side opposite from γ-NTP.

Exchange of phosphoryl groups in the complex CK-catalyzed reaction was analyzed as a first-order exchange reaction

$$PCr \overset{k}{\leftrightarrow} ATP \qquad (2)$$

In this model, the unidirectional pseudo-first-order rate con-

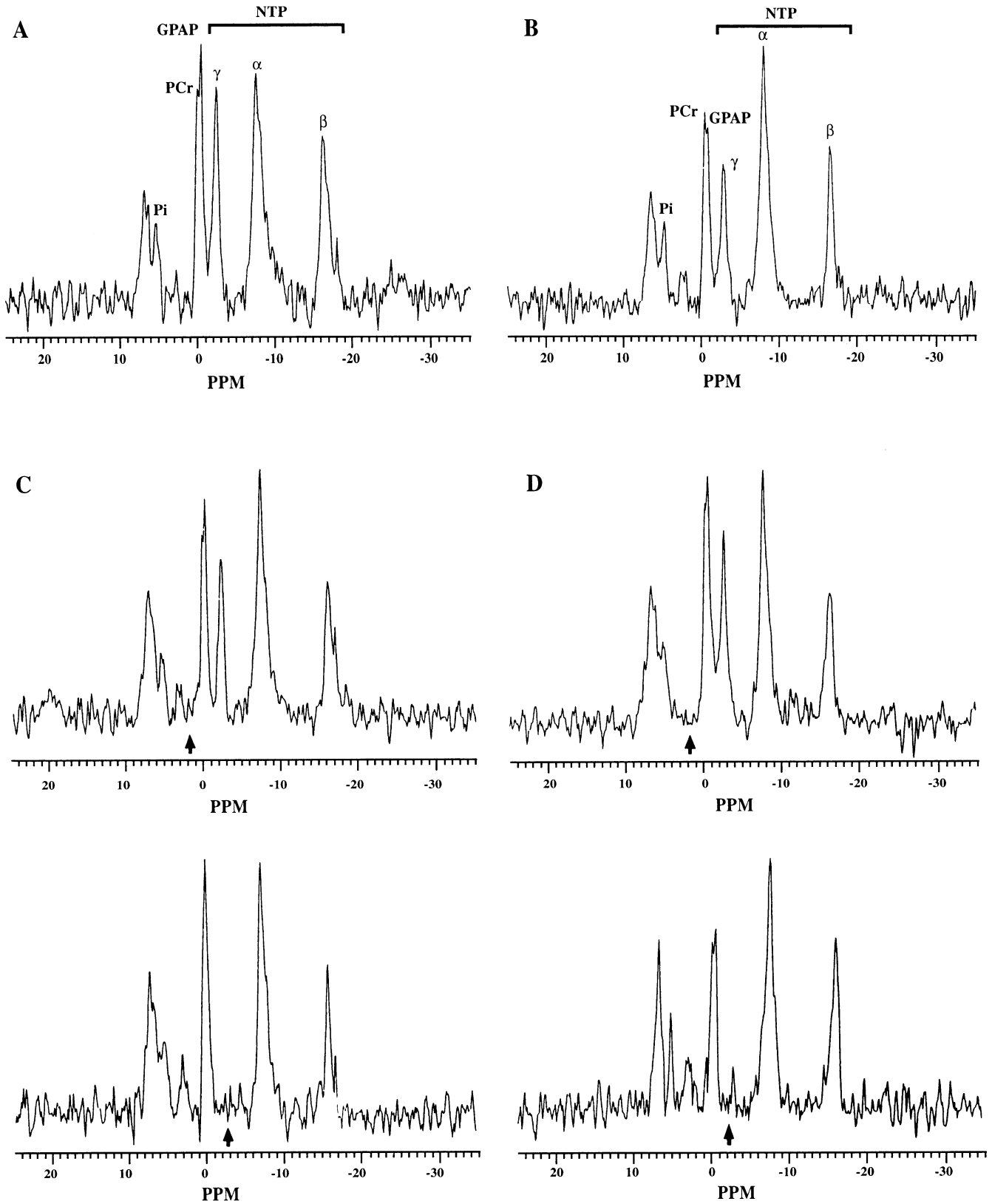

Fig. 1. ^{31}P nuclear magnetic resonance (^{31}P-NMR) brain spectra (*A* and *B*) and saturation transfer (ST) experiments (*C* and *D*) acquired before (*A* and *C*) and during (*B* and *D*) pentylenetetrazole (PTZ)-induced seizures in a mouse fed guanidinopropionic acid (GPA) for 3 wk. Spectra were partially saturated [interpulse interval (RD) of 2 s] and obtained as described in METHODS. The 2 phosphagen peaks [phosphocreatine (PCr) and phosphorylated GPA (GPAP)], the 3 NTP peaks, and the P_i peak are labeled. *C* and *D*: seizure spectrum and ST experiment were acquired 12 min after PTZ injection. ST experiments were acquired and the results were analyzed as described in METHODS with a saturation pulse (indicated by arrows) placed either on γ-NTP frequency (*bottom*) or at an equal distance on opposite side of PCr peak (*top*). ppm, Parts per million.

Table 1. *Creatine kinase pseudo-first-order reaction rate constants and reactant concentrations in brains of control mice and mice fed GPA for at least 3 wk*

	Phosphagen-to-β-NTP Ratio	n	pH	n	k, s^{-1}	n
Control	2.21 (0.68–4.26)	25	7.11 (6.70–7.26)	24	0.27 (0.03–0.44)	25
GPA	1.51 (0.87–3.38)	24	7.17 (6.97–7.40)	21	0.09 (0–0.26)	25
P	0.13		0.07		≤0.0001	

Each value is median of range shown in parentheses; n = no. of experiments. Rate constants (k) and reactant concentrations were measured in vivo by ^{31}P nuclear magnetic resonance (^{31}P-NMR) in brains of control and β-guanidinopropionic acid (GPA)-fed mice. The phosphagen measurement included phosphocreatine (PCr) for controls and PCr + phosphorylated GPA (GPAP) for GPA-fed mice. P values were calculated for difference between control and GPA groups.

stant (k) is equal to the ratio of chemical flux (J) to the substrate concentration (2). Use of the first-order reaction simplification does not imply that the CK-catalyzed reaction is first order. The k for the forward direction was calculated from the NMR study as

$$k = J/[\text{PCr}] = 1/(T_1)_{\text{PCr}} \times (\text{Mo} - \text{Ms})/\text{Ms} \quad (3)$$

where T_1 is the longitudinal relaxation time of PCr in the absence of chemical exchange, Ms is the PCr signal in the presence of γ-NTP saturation, and Mo is the PCr signal obtained with the saturating pulse on the opposite side of PCr (Fig. 1). A value of 3 s was used for the T_1 of PCr, as previously measured in a progressive saturation experiment (17). The same T_1 was assumed for GPAP.

Total brain CK activity. Total brain CK activity was measured in a separate set of control mice and mice fed GPA for 6 or 12 wk as previously described (23). Animals were anesthetized with urethane and decapitated. The cerebral hemispheres were removed and immediately placed in liquid N_2. While still frozen, the brains were weighed and then homogenized with a Potter homogenizer in 2–3 ml of ice-cold water. The homogenates remained in the ice bath for another 30 min to allow mitochondrial swelling. Triton (0.05%) and NaH_2PO_4 (50 mM final concentration) were added to osmotically break up mitochondria and extract the Mi-CK. The homogenate was allowed to sit on ice for 2–3 h. Homogenate samples were then centrifuged for 25 min at 11,000 g (10,000 revolutions/min) in an SS 34 rotor (Sorvall RC 24 centrifuge). The resulting supernatant was removed in 5-μl aliquots for pH

Table 2. *Total CK activity measured in cerebral hemisphere homogenates from control mice and mice fed GPA for 6 or 12 wk*

	Feeding Duration, wk	Total CK Activity, enzyme units/mg protein	n
Control	6	7.37 (2.48–48.9)	6
GPA	6	20.47 (3.98–42.91)*	6
Control	12	12.68 (4.74–21.9)	4
GPA	12	25.0 (22–31.84)†	5

Each value is median of range shown in parentheses; n = no. of brains. CK, creatine kinase. *P = 0.34 and †P = 0.01, calculated for difference between control and GPA groups for each feeding duration.

Table 3. *CK pseudo-first-order reaction rate constants and reactant concentrations in brains of control mice and mice fed creatine for at least 3 wk*

	PCr-to-β-NTP Ratio	n	pH	n	k, s^{-1}	n
Control	1.44 (1.16–1.76)	21	7.07 (6.97–7.27)	24	0.25 (0.09–0.52)	21
Creatine	1.59 (1.11–2.13)	20	7.09 (7.01–7.21)	20	0.25 (0–0.63)	20
P	0.02		0.31		0.95	

Each value is median of range shown in parentheses; n = no. of experiments. CK pseudo-first-order reaction k values and reactant concentrations were measured in vivo by ^{31}P-NMR. P values were calculated for difference between control and creatine groups.

stat measurements (55). Background kinase activity was measured in the presence of 4 mM ADP. Total CK activity was measured in the presence of 10 mM PCr. The CK activities are expressed per milligram protein. Protein content was measured in each sample (31).

Statistics. Differences between baseline results in control and experimental mice were examined with Wilcoxon's ranked sum test. Statistical significances for changes in reactant

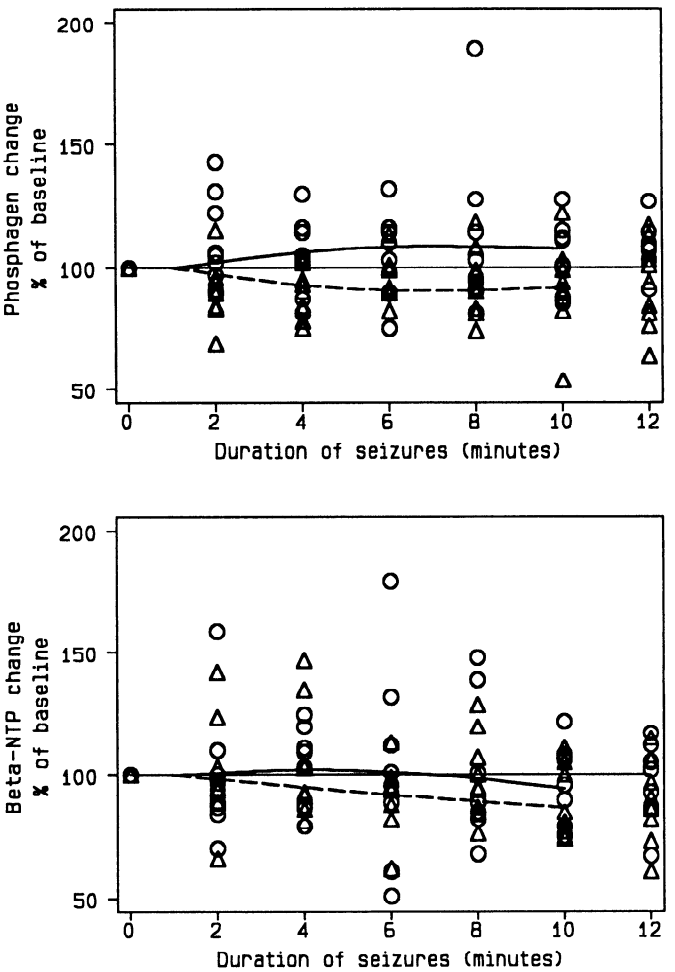

Fig. 2. Changes in brain total phosphagen (PCr in controls, PCr plus GPAP in GPA-fed mice; *top*) and NTP (*bottom*) concentrations during 12 min of PTZ-induced seizures in 6–8 control (△ and dashed lines) and GPA-fed (○ and solid lines) mice. All values are expressed as a percentage of NMR spectral peak areas measured before PTZ. Differences between control and GPA-fed mice in the changes during seizures were significant only for phosphagen ($P < 0.001$).

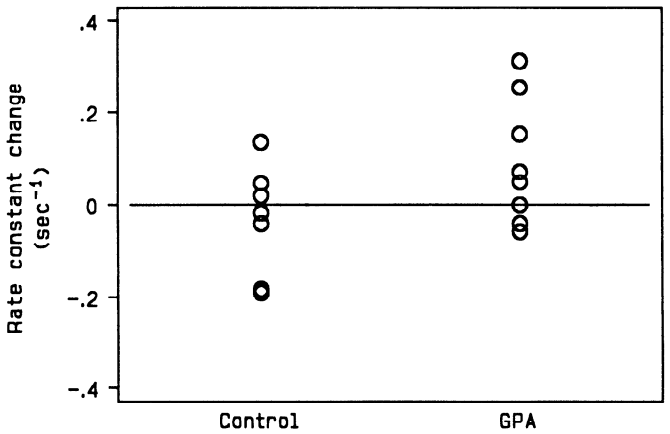

Fig. 3. Changes in creatine kinase (CK)-catalyzed pseudo-first-order reaction rate constants measured in brains of control and GPA-fed mice after 12 min of PTZ-induced seizures. Differences were calculated for rate constants during seizures minus values before seizures. Values for GPA-fed animals were calculated from overlapping PCr and GPAP peaks, giving measures of total phosphagen exchange for each group. Rate constants, which were higher before seizures in controls, were not different in the 2 groups during seizures.

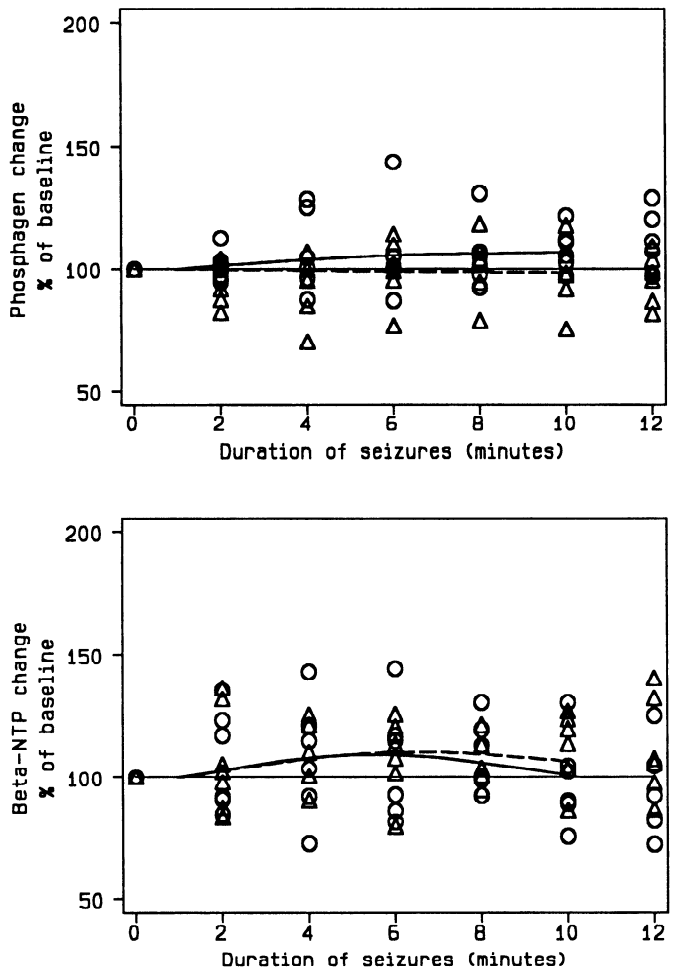

Fig. 4. Changes in brain PCr (*top*) and NTP (*bottom*) concentrations during 12 min of PTZ-induced seizures in 6 control (△ and dashed line) and creatine-fed (○ and solid line) mice. All values are expressed as a percentage of NMR spectral peak areas before PTZ. No differences between the groups were seen.

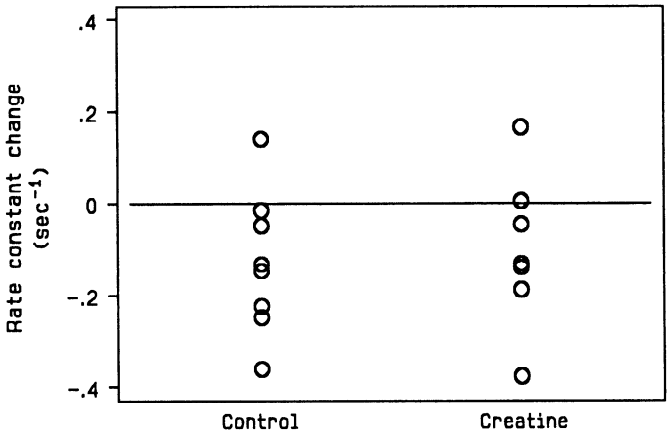

Fig. 5. Changes in CK-catalyzed pseudo-first-order reaction rate constants in brains of 6 control and 6 creatine-fed mice before and after 12 min of PTZ-induced seizures. Differences were calculated for rate constants measured during seizures minus values before seizures. Rate constants were not different between the groups either during or before seizures.

concentrations and reaction rates in each group during hypoxia or seizures were calculated with analysis of covariance on ranked data (nonparametric analysis of covariance). Nonparametric tests were used because of the relatively small numbers available for these comparisons. Smoothed curves connecting the median y values are shown in Figs. 2, 4, 6, and 8. Analyses and graphics were performed with Stata software (Stata, College Station, TX).

RESULTS

In a preliminary experiment, five GPA-fed mice and five controls were weighed twice each week. During the first 2 wk, the GPA-fed mice lost 10% of their body weight. The initial weight was regained within 6 wk of starting GPA feedings (median wt of 25 g, range of 24.5–26 g). During the first 6 wk of feedings, controls gained 20% of their body weight from a median of 25 (24–26 g) to 30 g (28–31 g). The fur was thinner in the GPA-fed mice. By observation, activity was the same in the two groups.

As shown in Table 1, ^{31}P-NMR measures of brain CK reactant concentration ratios and pseudo-first-order reaction k values in GPA-fed mice were similar to those previously reported (18). Total phosphagen-to-NTP ratios were lower than PCr-to-NTP ratios in controls, a difference that approached significance. As estimated from peak heights, brain PCr and GPAP concentrations were approximately equal (median PCr/GPAP of 1.03, range of 0.63–1.72). Median brain pH was more alkaline in GPA-fed mice than in controls, a difference that only approached significance. The CK reaction k value

Table 4. *Deaths during and immediately after hypoxia in control mice and mice fed GPA for at least 3 wk*

	Inspired Oxygen		
	6%	4%	2%
Control	0/8	2/6	4/7
GPA	0/8	0/6	1/6

Ratios are deaths/total number in each group.

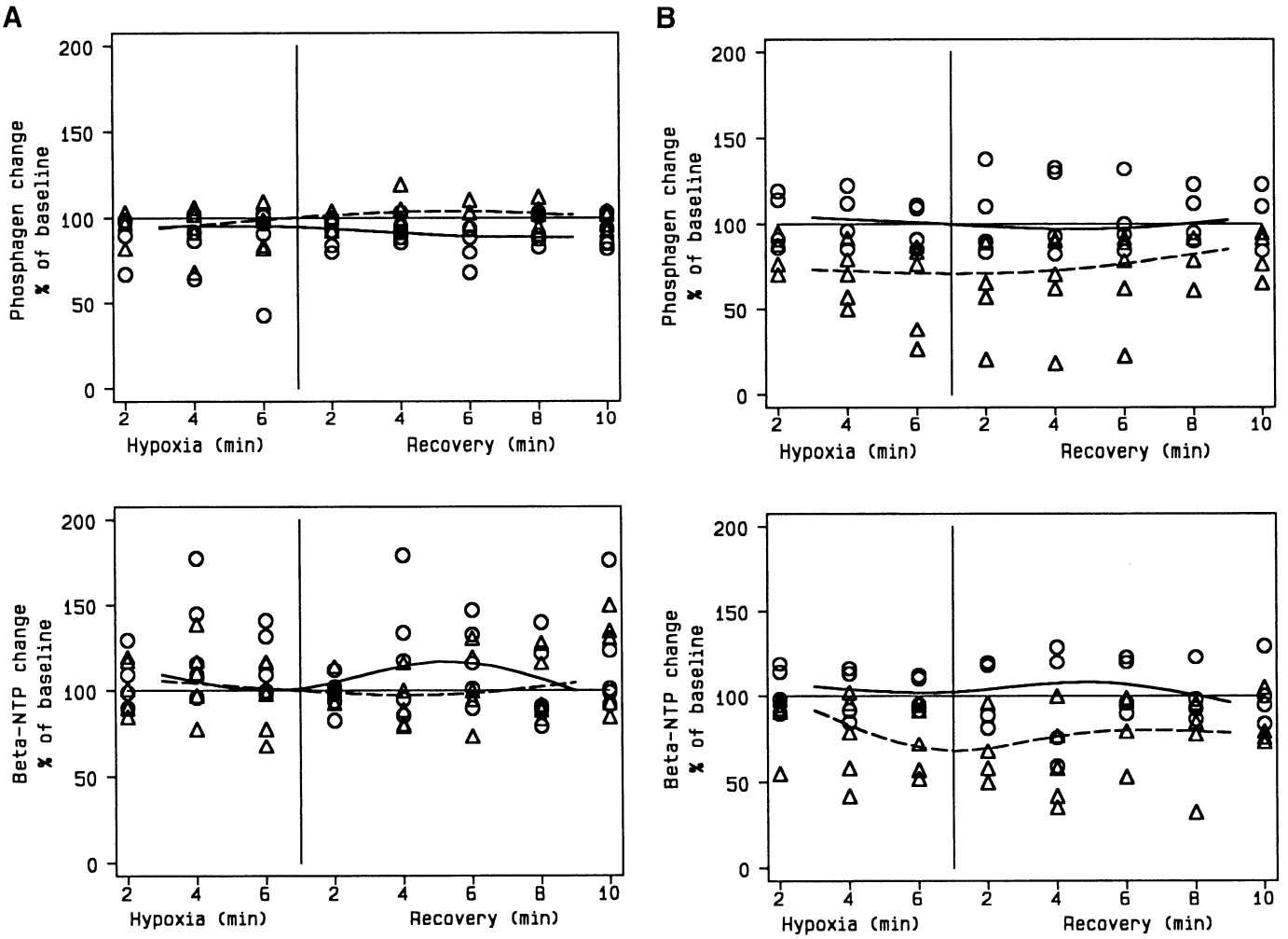

Fig. 6. Changes in brain PCr or PCr plus GPAP (*top*) and in NTP (*bottom*) concentrations during hypoxia and after recovery from hypoxia in control mice (△ and dashed lines) and mice fed GPA (○ and solid lines) for at least 3 wk. Results are shown for animals breathing 6% (*A*) or 4% (*B*) O_2 for 7 min and then breathing room air. All values are concentrations determined from ^{31}P-NMR spectra and expressed as a percentage of prehypoxic peak areas for the 2-s RD spectra. Significantly larger decreases in brain CK reactants were seen in control compared with GPA-fed mice ($P < 0.001$ for PCr at 6% and for PCr and NTP at 4% O_2).

was much lower in GPA-fed mice. As estimated by peak heights, the phosphoryl exchange between PCr and ATP was 70% lower in GPA-fed mice than in controls (results not shown), a difference similar to that previously found by the same method (18). The CK-catalyzed k values were much higher for transfer of phosphoryl groups from PCr than from GPAP, with very little change in GPAP peak height seen in the γ-NTP-saturated spectra (Fig. 1).

Total CK activity in cerebral hemispheres doubled after 6 and 12 wk of GPA feedings (Table 2). The increase in CK activity was highly significant after 12 wk. The doubling of brain total CK activity has also been found in GPA-fed rats (35). In the rat, the increase is predominantly in B-CK with only a small increase seen in Mi-CK (35).

Baseline brain CK reactants and k values were similar in creatine-fed mice and controls (Table 3). Unlike in GPA-fed mice, PCr/NTP showed a consistent, small increase in creatine-fed mice. The pH and CK-catalyzed reaction k values were unchanged in creatine-fed animals. The CK activities were not measured in creatine-fed mice.

Seizures. No GPA-fed mice or controls died during the 15–20 min of PTZ-induced seizures. Electrocortical seizure activity was more prominent in GPA-fed mice than in controls. Three of four GPA-fed mice showed prolonged bursts of sharp waves or spikes, and one showed only isolated spikes. Two control mice showed briefer periods of spike bursts or sharp waves, and one showed only isolated sharp waves. All EEG changes occurred within 3–5 min of PTZ injections and continued throughout the recordings.

Typical spectra and ST experiments from a GPA-fed mouse before and during seizures are shown in Fig. 1. There were no significant changes in brain ATP, as deduced from the β-NTP signal, in either GPA-fed mice or controls during seizures (Figs. 1 and 2). Brain total phosphagen concentration increased during seizures in the GPA-fed mice. As estimated by peak heights, the

brain phosphagen increase was due to an increase in PCr, whereas GPAP decreased (result not shown). In brain of control mice, the PCr concentration decreased 10–20%. The difference in phosphagen changes during seizures in GPA-fed mice compared with controls was highly significant ($P < 0.001$). Brain pH did not change in controls or in GPA-fed mice, remaining more alkaline in the GPA-fed mice throughout the period of seizures.

The CK-catalyzed reaction k values in GPA-fed mice increased more than in controls during seizures (Fig. 3). In five of seven GPA-fed mice the k values increased at least 40%. Median k values, which were significantly higher in controls than in GPA-fed mice before seizures (Table 1), were not different in the two groups during seizures.

No creatine-fed mice died during seizures. There were no significant changes in PCr or NTP concentration in either group (Fig. 4). In both groups, the CK reaction rates decreased during seizures (Fig. 5).

Hypoxia. Deaths during or after breathing hypoxic gas mixtures were much less frequent in GPA-fed mice than in controls (Table 4). Only 1 of the 12 GPA-fed mice died breathing 4% and 2% O_2 compared with 5 deaths in 13 controls ($P < 0.05$). There were no deaths in either group exposed to 6% O_2.

Losses of brain phosphagen and ATP during and after hypoxia were also less in GPA-fed mice than in controls (Fig. 6). No changes in phosphagen or NTP were seen in either group when they were breathing 6% O_2. Total phosphagen did not change in GPA-fed mice, whereas PCr decreased ~25% in controls that were breathing 4% O_2 ($P < 0.001$ for the difference between controls and GPA-fed mice). By comparing changes in peak heights, the smaller losses of total phosphagen in GPA-fed mice reflected stable PCr and GPAP during hypoxia (results not shown). At 4% O_2, there were no NTP changes in GPA-fed mice, whereas the NTP signal decreased ~20% in the controls, indicating a 25–30% decrease in brain ATP ($P < 0.001$). In GPA-fed mice breathing 2% O_2, total phosphagen showed a transient 25% decrease, whereas ATP decreased ~20% during and after the hypoxic period (results not shown). The PCr and NTP decreases were the same in GPA-fed mice and surviving controls that were breathing 2% O_2. The P_i peak usually broadened during hypoxia, but there were no consistent changes in the chemical shift in either group.

Changes in the CK-catalyzed reaction k values are shown in Fig. 7. Although the numbers were small, k values for CK increased after 6 and 2% O_2 in the GPA-fed animals but not in controls. The k values for CK, which were much higher in controls than in GPA-fed mice before hypoxia (Table 1), were the same in the two groups after hypoxia. At 4% O_2, the k values for CK decreased in controls and did not change in GPA-fed mice.

The frequency of deaths and losses of PCr and ATP during hypoxia were the same in creatine-fed mice and controls (Table 5 and Fig. 8). The CK reaction rates decreased in both groups after 6% O_2 (Fig. 9).

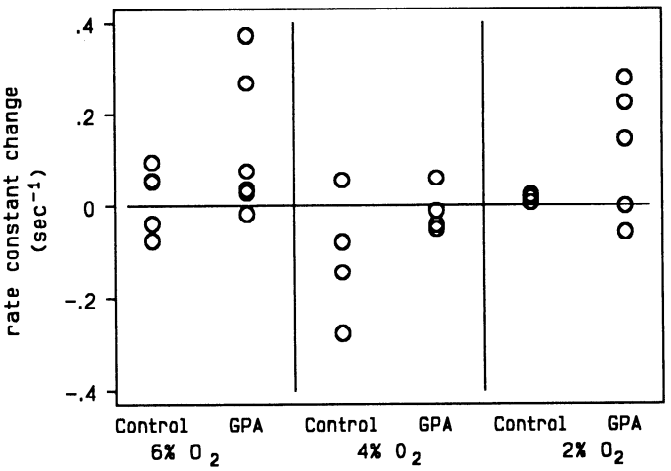

Fig. 7. Changes in brain CK-catalyzed pseudo-first-order reaction rate constants before and after hypoxia are shown for control and GPA-fed mice. Each value is difference between rate constant after hypoxia and that measured before hypoxia as described in METHODS. Hypoxic gas mixtures were 6%, 4%, or 2% O_2. Rate constants for GPA-fed mice were calculated from integration of the overlapping PCr and GPAP peaks and thus represent total flux from phosphagen to ATP. Differences in flux changes in the 2 groups were not significant.

DISCUSSION

The results of GPA feedings support the proposed compartmentation of the creatine-CK-PCr system in brain (21, 23, 28, 50). Unlike muscle, where GPA completely replaces creatine, GPA feedings result in equal brain GPAP and PCr concentrations. The partial replacement of PCr has suggested two creatine compartments, one in which creatine is replaced by GPA and one in which creatine is not decreased and possibly is not accessible to GPA (18). In this model, the lower brain CK-catalyzed reaction rate, measured with PCr as substrate, is the property of the GPA-inaccessible compartment. An assumption is that most of the GPA in brain is phosphorylated and thus is seen as GPAP by NMR.

An anatomic compartmentation of creatine-CK-PCr systems has been recently proposed that is based on ^{31}P-NMR one-dimensional chemical shift imaging studies of the piglet brain (50). The creatine-CK-PCr system, which predominates in cerebral gray matter, is characterized by low PCr-to-NTP and PCr-to-creatine ratios, the presence of B-CK and Mi-CK isoenzymes, and relatively slow CK-catalyzed reaction rates in vivo (21, 23, 57). These characteristics are similar to reactant ratios and isoenzymes seen in smooth muscle (54). In contrast, white matter shows much higher PCr-to-

Table 5. *Deaths during and immediately after hypoxia in control mice and mice fed creatine for at least 3 wk*

	Inspired Oxygen		
	6%	4%	2%
Control	2/7	5/7	3/3
Creatine	2/7	5/7	3/3

Ratios are deaths/total number in each group.

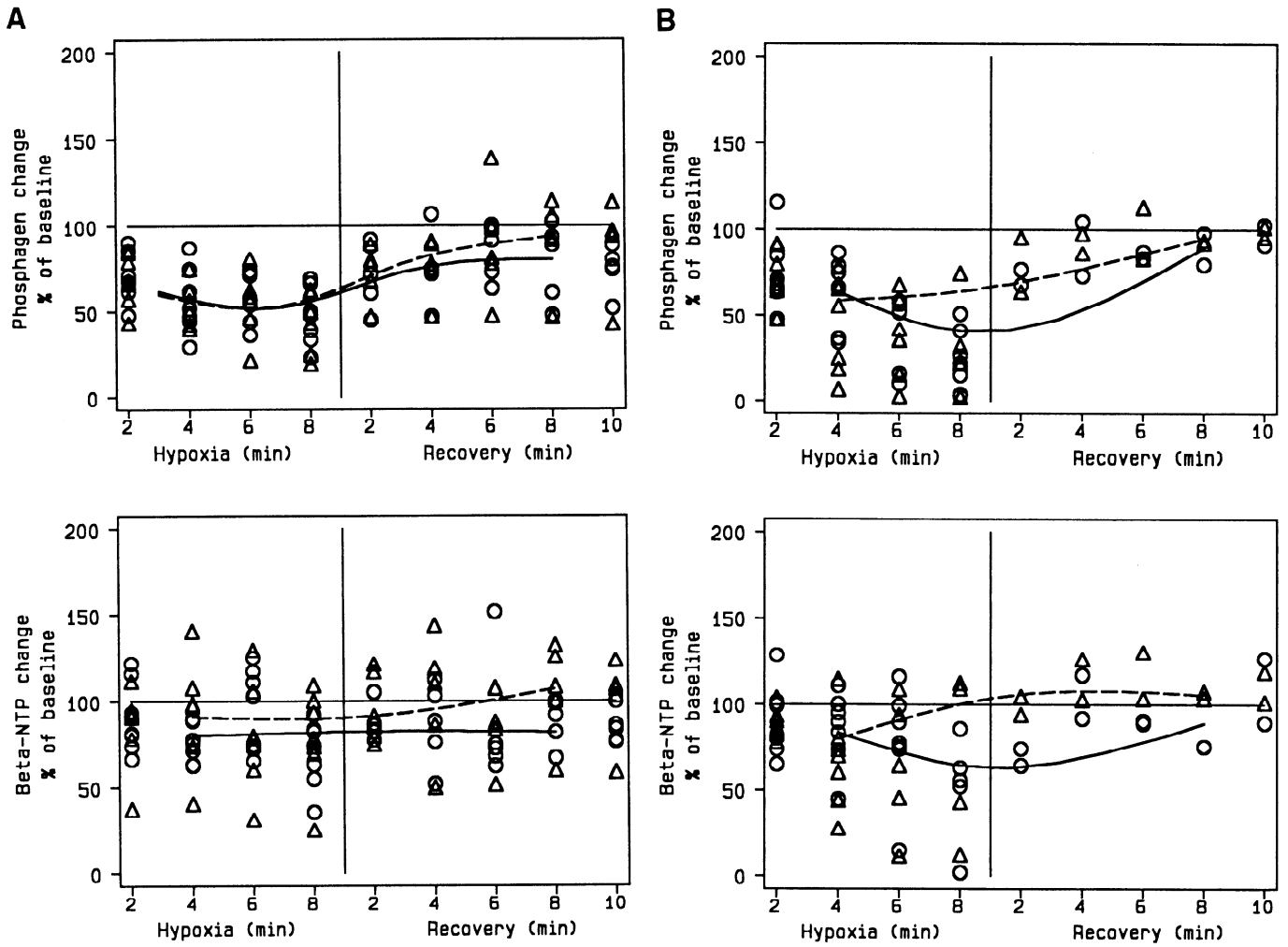

Fig. 8. Changes in brain PCr and NTP concentrations during hypoxia and after recovery from hypoxia in control mice (△ and dashed lines) and mice fed creatine (○ and solid lines) for at least 3 wk. Results are shown for mice breathing either 6% (*A*) or 4% (*B*) O_2 for 7 min and then room air. All values are concentrations determined from ^{31}P-NMR spectra and expressed as a percentage of the prehypoxic peak areas for 2-s RD spectra. There were no differences between creatine-fed and control mice.

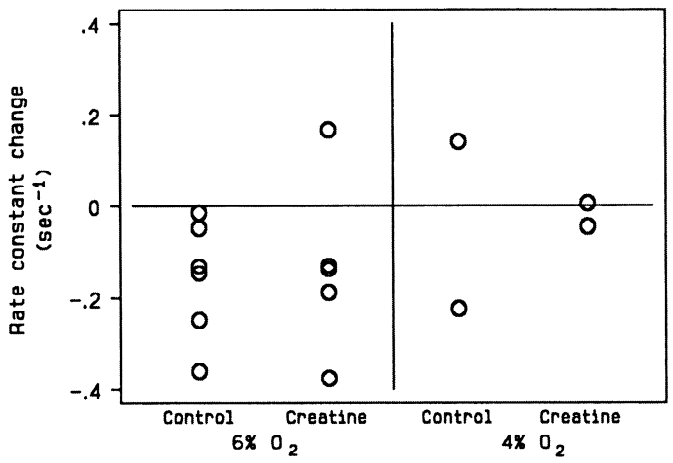

Fig. 9. Changes in CK-catalyzed pseudo-first-order reaction rate constants in control and creatine-fed mice after breathing 6% or 4% O_2 concentrations. There were no differences between the 2 groups of mice.

NTP and PCr-to-creatine ratios, higher in vivo CK-catalyzed reaction rates, and little or no Mi-CK. The CK system in white matter is like that found in skeletal muscle (29). The effects of GPA feedings are consistent with the analog blocking creatine transport and GPAP replacing PCr predominantly in white matter. Thus the remaining PCr shows properties of gray matter including low PCr-to-NTP ratios and CK-catalyzed fluxes. A high concentration of the creatine transporter is present in white matter (40). The creatine, which is not replaced by GPA, may be synthesized and not transported in brain (8).

As seen in previous studies, brain ATP synthesis is closely coupled to the increased energy demand during seizures in all three groups of mice (19, 37, 60). With the assumption that both PCr and GPAP are substrates for the CK reaction in brain, the increase in PCr and decrease in GPAP indicate very different physiological functions for the two phosphagens in the GPA-fed mice. The decrease in GPAP, as a hypothetical marker of

white matter phosphagen, suggests that the phosphagen analog functions as an energy reserve, the consumption of which is coupled to ATP synthesis. The cellular site may be the oligodendroglial processes (21). The increase in PCr, hypothetically in cortical gray matter, occurs despite the increased electrocortical activity and ATP turnover. Although the mechanisms for this novel observation are unknown, the increase in PCr could result from an uncoupling of the creatine-CK-PCr reaction from ATP synthesis at cellular sites where the PCr-to-creatine ratio is low and, therefore, probably far from equilibrium (21, 50). With the assumption that these phosphagen changes are from regional physiology, the PCr changes in control mice measured by surface coil-localized spectroscopy would be the sum of these opposite concentration changes during seizures.

Decreased brain GPAP during hypoxia supports the proposal that GPAP is a CK substrate in vivo. The hypothesis for the present studies was that the less effective substrate, GPAP, would increase hypoxic brain ATP loss. Although the number of animals studied was small, the enhanced survival of GPA-fed mice and the stable brain ATP during hypoxia are clear. Analogous in vitro effects of creatine are seen in hypoxic hippocampal slices, in which creatine loading prolongs synaptic transmission and decreases cell injury (5, 58). These observations suggested a second hypothesis in which ATP concentration is supported by increased PCr and GPA may be acting as additional, slowly metabolized phosphagen. However, this hypothesis was not supported by results of creatine feedings, which increase the brain PCr-to-NTP ratio but do not enhance hypoxic survival or stabilize brain ATP. Finally, stable brain ATP and enhanced hypoxic survival may be due in part to a myocardial effect in which PCr is fully replaced by GPAP (43).

Changes in CK isoenzymes and ATP metabolic pathways, which may be adaptations to GPA or to creatine/PCr depletion, most likely contribute to the enhanced hypoxic survival and maintenance of brain ATP during hypoxia and seizures. In skeletal muscle, where GPA almost completely replaces creatine, metabolic adaptations are similar to those seen in exercise (10, 13, 32–35, 43–45). Functionally, contractile properties of the muscle fibers are maintained (34). As proposed above, the GPA effects in brain may be mostly in white matter in which the creatine-CK-PCr system is similar to that of skeletal muscle. In brain, in which PCr is only partially depleted, total CK increases rather than decreases as in muscle. The increase is in B-CK in contrast to the decrease in B-CK in skeletal muscle (35). If confined to white matter, the small increase in whole brain Mi-CK may reflect a large regional increase, since white matter has only a small Mi-CK activity (23).

Guanidino compounds will be important in studying the physiology of the creatine-CK-PCr system in brain energetics, plasticity of ATP metabolism, and pathogenesis of inborn errors of human energy metabolism. Feeding GPA alters ATP metabolic pathways in brain and alters ATP regulation during hypoxia and seizures. These metabolic changes, the signals for which are not known, demonstrate a plasticity of brain energy metabolism. This plasticity may be critical in understanding adaptation to physiological conditions of increased energy requirements such as learning and development as well as pathological states such as hypoxia and seizures. The clinical relevance of these studies is also seen in the parallel upregulation of sarcomeric Mi-CK and the morphology of the intramitochondrial inclusions in skeletal muscle of rats fed GPA and in patients with ragged red fiber myopathies (9, 15, 39, 47, 53). The brains of patients with mitochondrial encephalomyopathies, like the GPA-fed rodents, do not show mitochondrial morphological changes.

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REFERENCES

1. **Ackerman, J., T. Grove, G. Wong, and D. Gadian.** Mapping of metabolites in whole animals by 31P-NMR using surface coils. *Nature* 283: 167–170, 1980
2. **Alger, J., and R. Shulman.** NMR studies of enzymatic rates in-vitro by magnetization transfer. *Q. Rev. Biophys.* 17: 83–124, 1984.
3. **Bessman, S., and P. Geiger.** Transport of energy in muscle: the phosphorylcreatine shuttle. *Science* 211: 448–452, 1981.
4. **Blennow, G., J. Folbergrova, B. Nilsson, and B. Siesjo.** Cerebral metabolic and circulatory changes in the rat during sustained seizures induced by D,L-homocysteine. *Brain Res.* 179: 129–146, 1979.
5. **Carter, A., E. Muller, U. Pschorn, and W. Stransky.** Preincubation with creatine enhances levels of creatine phosphate and prevents anoxic damage in rat hippocampal slices. *J. Neurochem.* 64: 2691–2699, 1995.
6. **Chapman, A., E. Westerberg, and B. Siesjo.** The metabolism of purine and pyrimidine nucleotides during insulin-induced hypoglycemia and recovery. *J. Neurochem.* 36: 179–186, 1981.
7. **Chevli, R., and C. Fitch.** Beta-guanidinopropionate and phosphorylated beta-guanidinopropionate as substrates for creatine kinase. *Biochem. Med.* 21: 162–167, 1979.
8. **Defalco, A., and R. Davies.** The synthesis of creatine by the brain of the intact rat. *J. Neurochem.* 7: 308–312, 1961.
9. **DeTata, V., G. Cavallini, M. Pollera, Z. Gori, and E. Bergamini.** The induction of mitochondrial myopathy in the rat by feeding beta-guanidinopropionic acid and the reversibility of the induced mitochondrial lesions: a biochemical and ultrastructural investigation. *Int. J. Exp. Pathol.* 74: 501–509, 1993.
10. **Fitch, C., M. Jellinek, and E. Mueller.** Experimental depletion of creatine and phosphocreatine from skeletal muscle. *J. Biol. Chem.* 249: 1060–1063, 1974.
11. **Fitch, C., R. Shields, W. Payne, and J. Dacus.** Creatine metabolism in skeletal muscle. III. Specificity of the creatine entry process. *J. Biol. Chem.* 243: 2024–2029, 1968.

12. **Forsen, S., and R. Hoffmann.** Study of moderately rapid chemical exchange reactions by means of nuclear magnetic double resonance. *J. Chem. Phys.* 39: 2892–2901, 1963.
13. **Freyssenet, D., P. Berthon, A Geyssant, and C. Denis.** ATP synthesis kinetic properties of mitochondria isolated from the rat extensor digitorum longus muscle depleted of creatine with beta-guanidinopropionic acid. *Biochim. Biophys. Acta* 1186: 232–236, 1994.
14. **Gadian, D.** *Nuclear Magnetic Resonance and its Applications to Living Systems.* New York: Oxford Univ. Press, 1982, p. 197.
15. **Gori, Z., V. DeTata, M. Pollera, and E. Bergamini.** Mitochondrial myopathy in rats fed with a diet containing beta-guanidinopropionic acid, an inhibitor of creatine entry in muscle cells. *Br. J. Exp. Pathol.* 69: 639–650, 1988.
16. **Hiramatsu, T., R. Jonas, T. Miura, A. du Plessis, M. Tanji, J. Forbess, M. Tsuji, and D. Holtzman.** Cerebral metabolic recovery from deep hypothermic circulatory arrest after treatment with arginine and nitro-arginine-methylester. *J. Thorac. Cardiovasc. Surg.* In press.
17. **Holtzman, D., E. McFarland, D. Jacobs, M. Offutt, and L. Neuringer.** Maturational increase in mouse brain creatine kinase reaction rates shown by phosphorus magnetic resonance. *Dev. Brain Res.* 58: 181–188, 1991.
18. **Holtzman, D., E. McFarland, T. Moerland, J. Koutcher, M. Kushmerick, and L. Neuringer.** Brain creatine phosphate and creatine kinase in mice fed an analogue of creatine. *Brain Res.* 483: 68–77, 1989.
19. **Holtzman, D., R. Meyers, I. Khait, and F. Jensen.** Brain creatine kinase reaction rates and reactant concentrations during seizures in developing rats. *Epilepsy Res.* In press.
20. **Holtzman, D., R. Meyers, E. O'Gorman, and T. Wallimann.** Cerebral creatine kinase reaction rates and reactants during hypoxia and seizures in mice fed a creatine analog (Abstract). *J. Neurochem.* 66: S95, 1996.
21. **Holtzman, D., R. Mulkern, M. Tsuji, C. Cook, and R. Meyers.** Phosphocreatine and creatine kinase systems in gray and white matter of the developing piglet brain. *Dev. Neurosci.* 18: 535–542, 1996.
22. **Holtzman, D., M. Tsuji, L. Neuringer, and D. Jacobs.** Creatine kinase reaction rates in the cyanide-poisoned mouse brain. *J. Cereb. Blood Flow Metab.* 13: 153–161, 1993.
23. **Holtzman, D., M. Tsuji, T. Wallimann, and W. Hemmer.** Functional maturation of creatine kinase in rat brain. *Dev. Neurosci.* 15: 261–270, 1993.
24. **Hossle, J., J. Schlegel, G. Wegmann, M. Wyss, P. Bohlen, J. Eppenberger, T. Wallimann, and J. Perriard.** Distinct tissue-specific mitochondrial creatine kinases from chicken brain and striated muscle with conserved CK framework. *Biochem. Biophys. Res. Commun.* 151: 408–416, 1988.
25. **Howse, D. C., J. J. Caronna, T. E. Duffy, and F. Plum.** Cerebral energy metabolism, pH, and blood flow during seizures in the cat. *Am. J. Physiol.* 227: 1444–1451, 1974.
26. **Jacobs, H., W. Heldt, and M. Klingenstein.** High activity of CK in mitochondria from muscle and brain. Evidence for a separate mitochondrial isoenzyme of CK. *Biochem. Biophys. Res. Commun.* 16: 516–521, 1964.
27. **Jacobus, W., and A. Lehninger.** Creatine kinase of rat heart mitochondria. Coupling of creatine phosphorylation to electron transport. *J. Biol. Chem.* 248: 4803–4810, 1973.
28. **Kaldis, P., W. Hemmer, E. Zanolla, D. Holtzman, and T. Wallimann.** "Hot spots" of creatine kinase localization in the brain: cerebellum, hippocampus and choroid plexus. *Dev. Neurosci.* 18: 542–554, 1996.
29. **Kushmerick, M. J.** Energetics of muscle contraction. In: *Handbook of Physiology. Skeletal Muscle.* Bethesda, MD: Am. Physiol. Soc., 1983, sect. 10, chapt. 7, p. 189–236.
30. **Lolly, R., W. Balfour, and F. Samson.** The high energy phosphates in developing brain. *J. Neurochem.* 7: 289–297, 1961.
31. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193: 265–275, 1951.
32. **Meyer, R. A., T. R. Brown, B. L. Krilowicz, and M. J. Kushmerick.** Phosphagen and intracellular pH changes during contraction of creatine-depleted rat muscle. *Am. J. Physiol.* 250 (*Cell Physiol.* 19): C264–C274, 1986.
33. **Moerland, T. S. , and M. J. Kushmerick.** Contractile economy and aerobic recovery metabolism in skeletal muscle adapted to creatine depletion. *Am. J. Physiol.* 267 (*Cell Physiol.* 36): C127–C137, 1994.
34. **Moerland, T. S., N. G. Wolfe, and M. J. Kushmerick.** Administration of a creatine analogue induces isomyosin transitions in muscle. *Am. J. Physiol.* 257 (*Cell Physiol.* 26): C810–C816, 1989.
35. **O'Gorman, E., G. Beutner, T. Wallimann, and D. Brdiczka.** Differential effects of creatine depletion on the regulation of enzyme activities and on creatine-stimulation mitochondrial respiration in skeletal muscle, heart, and brain. *Biochim. Biophys. Acta* 1276: 161–170, 1996.
36. **Petroff, O., J. Prichard, K. Behar, J. Alger, J. Den Hollander, and R. Shulman.** Cerebral intracellular pH by 31P nuclear magnetic resonance spectroscopy. *Neurology* 35: 781–788, 1985.
37. **Petroff, O., J. Prichard, K. Behar, J. Alger, and R. Shulman.** In vivo phosphorus nuclear magnetic resonance spectrosopy in status epilepticus. *Ann. Neurol.* 16: 169–177, 1984.
38. **Prichard, J., J. Alger, K. Behar, O. Petroff, and R. Shulman.** Cerebral metabolic studies in vivo by 31P-NMR. *Proc. Natl. Acad. Sci. USA* 80: 2748–2751, 1983.
39. **Riesinger, I., C. Haas, and T. Wallimann.** Mitochondrial inclusions induced by feeding a creatine analogue exhibit a high density of mitochondrial creatine kinase (EBEC short reports). *Biochim. Biophys. Acta* 7: 140–146, 1992.
40. **Saltarelli, M., A. Bauman, K. Moore, C. Bradley, and R. Blakely.** Expression of the rat brain creatine transporter in situ and in transfected HeLa cells. *Dev. Neurosci.* 18: 524–534, 1996.
41. **Sauter, A., and M. Rudin.** Determination of creatine kinase kinetic parameters in rat brain by NMR magnetization transfer. *J. Biol. Chem.* 268: 13166–13171, 1993.
42. **Shoubridge, E., R. Challis, D. Hayes, and G. Radda.** Biochemical adaptation in the skeletal muscle of rats depleted of creatine with the substrate analogue beta-guanidinopropionic acid. *Biochem. J.* 232: 125–131, 1985.
43. **Shoubridge, E., F. Jeffry, J. Keogh, G. Radda, and A. Seymour.** Creatine kinase kinetics, ATP turnover, and cardiac performance in hearts depleted of creatine with the substrate analogue beta-guanidinopropionic acid. *Biochim. Biophys. Acta* 847: 25–32, 1985.
44. **Shoubridge, E., and G. Radda.** A 31P nuclear magnetic resonance study of skeletal muscle metabolism in rats depleted of creatine. *Biochim. Biophys. Acta* 805: 79–88, 1984.
45. **Shoubridge, E. A., and G. K. Radda.** A gated ^{31}P-NMR study of tetanic contraction in rat muscle depleted of phosphocreatine. *Am. J. Physiol.* 252 (*Cell Physiol.* 21): C532–C542, 1987.
46. **Siesjo, B.** *Brain Energy Metabolism.* Chichester, UK: Wiley, 1978, p. 607.
47. **Stadhouders, A., P. Jap, H. Winkler, H. Eppenberger, and T. Wallimann.** Mitochondrial creatine kinase: a major constituent of pathological inclusions seen in mitochondrial myopathies. *Proc. Natl. Acad. Sci. USA* 91: 5089–5093, 1994.
48. **Toda, N.** Nitric oxide and the regulation of cerebral arterial tone. In: *Nitric Oxide in the Nervous System*, edited by S. Vincent. New York: Academic, 1995, p. 207–227.
49. **Tsuji, M., E. Allred, F. Jensen, and D. Holtzman.** Phosphocreatine and ATP regulation in the hypoxic developing rat brain. *Dev. Brain Res.* 85: 192–200, 1995.
50. **Tsuji, M., R. Mulkern, C. Cook, R. Myers, and D. Holtzman.** Relative phosphocreatine and nucleoside triphosphate concentrations in cerebral gray and white matter measured in vivo by 31P nuclear magnetic resonance. *Brain Res.* 707: 146–154, 1996.
51. **Tsuji, M., H. Naruse, J. Volpe, and D. Holtzman.** Cerebral oxygenation and energy state in hypoxic piglets. *Pediatr. Res.* 37: 253–259, 1995.
52. **Walker, J.** Creatine: biosynthesis, regulation, and function. *Adv. Enzymol.* 50: 177–242, 1979.
53. **Wallace, D.** Diseases of the mitochondrial DNA. *Annu. Rev. Biochem.* 61: 1175–1212, 1992.

54. **Wallimann, T., and W. Hemmer.** Creatine kinase in non-muscle tissues and cells. *Mol. Cell. Biochem.* 133/134: 193–220, 1994.
55. **Wallimann, T., T. Schloesser, and H. Eppenberger.** Function of M-line bound creatine kinase as an intramyofibrillar ATP regenerator at the receiving end of the phosphorylcreatine shuttle in muscle. *J. Biol. Chem.* 259: 5238–5246, 1984.
56. **Wallimann, T., M. Wyss, D. Brdiczka, K. Nicolay, and H. Eppenberger.** Significance of intracellular compartmentation, structure, and function of creatine kinase isoenzymes for cellular energy homeostasis: the phospho-creatine circuit. *Biochem. J.* 281: 21–40, 1992.
57. **Whittingham, T., A. Douglas, and D. Holtzman.** Creatine and nucleoside triphosphates in rat cerebral gray and white matter. *Cerebr. Metab. Dis.* In press.
58. **Whittingham, T., and P. Lipton.** Cerebral synaptic transmission during anoxia is protected by creatine. *J. Neurochem.* 37: 1618–1621, 1981.
59. **Wyss, M., J. Smeitink, R. Wevers, and T. Wallimann.** Mitochondrial creatine kinase: a key enzyme of aerobic energy metabolism. *Biochim. Biophys. Acta* 1102: 119–166, 1992.
60. **Young, R., M. Osbakken, R. Briggs, S. Yagel, D. Rice, and S. Goldberg.** 31P-NMR study of cerebral metabolism during prolonged seizures in the neonatal dog. *Ann. Neurol.* 18: 14–20, 1985.
61. **Zweier, J., W. Jacobus, B. Korecky, and Y. Brandejs-Barry.** Bioenergetic consequences of cardiac phosphocreatine depletion induced by creatine analogue feeding. *J. Biol. Chem.* 266: 20296–20304, 1991.