Neuroscience 144 (2007) 1305–1313

DIFFERENT METABOLISM OF GLUTAMATERGIC AND GABAERGIC COMPARTMENTS IN SUPERFUSED HIPPOCAMPAL SLICES CHARACTERIZED BY NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

J. M. N. DUARTE,[a,b] R. A. CUNHA[a]* AND R. A. CARVALHO[a,b]

[a]*Centre for Neurosciences of Coimbra, Institute of Biochemistry, Faculty of Medicine, University of Coimbra, 3004-504 Coimbra, Portugal*

[b]*Department of Biochemistry, University of Coimbra, Apartado 3126, 3001-401 Coimbra, Portugal*

Abstract—We investigated intermediary metabolism using ^{13}C-glucose and ^{13}C-acetate tracers followed by ^{13}C-nuclear magnetic resonance (NMR) isotopomer analysis in rat hippocampal slice preparations, the most widely used preparation for electrophysiological studies. Slices displayed a stable metabolic activity over a wide range of superfusion periods in the absence or presence of 50 μM 4-aminopyridine (4AP), which triggers an intermittent burst-like neuronal firing. This caused an increase of tricarboxylic acid (TCA)-related amino acids (glutamate, aspartate and GABA) and shortened the time required to reach metabolic and isotopic steady state (3 h in the presence of 4AP and 7 h in its absence). ^{13}C-NMR isotopomer analysis revealed an increase in TCA flux in astrocytes and in GABA compartments greater than in putative glutamatergic neurons and the fitting of these data further indicated that the metabolic network in GABAergic and glutamatergic compartments has a different design and reacts differently to the stimulation by the presence of 4AP. These results show that ^{13}C-isotopomer analysis allows estimating metabolic parameters/fluxes under both steady- and non-steady-state metabolic conditions in hippocampal slices, opening the possibility of combining electrophysiological and metabolic studies in the same preparation.

Key words: hippocampal slices, ^{13}C-NMR, intermediary metabolism, isotopomer analysis.

^{13}C-nuclear magnetic resonance (NMR) spectroscopy is a powerful tool to investigate intermediary metabolism since it is able to simultaneously detect ^{13}C incorporation into molecules and the positions of ^{13}C incorporation within the same molecule (isotopomers). It has been used for studies of brain intermediary metabolism both *in vitro* and *in vivo* (e.g. Cerdán et al., 1990; Ebert et al., 2003; García-Espinosa et al., 2004; Gruetter, 2002; Künnecke et al., 1993; Zwingmann and Leibfritz, 2003), allowing following of the fate of labeling from ^{13}C-enriched substrates through particular metabolic pathways. This has allowed demonstrating that glucose is the main metabolic fuel although the energy requirements of cerebral tissue can be satisfied by the oxidation of other substrates such as ketone bodies (Badar-Goffer et al., 1990; Cerdán et al., 1990; Künnecke et al., 1993; Melo et al., 2006), lactate (Bouzier et al., 2000; Hassel and Brathe 2000; Tyson et al., 2003) and even fatty acids (Ebert et al., 2003; Kuge et al., 1995).

Brain metabolism has been mainly investigated in the brain or in cultured brain cells, but scarcely in hippocampal slices (Cohen et al., 1984; Whittingham et al., 1984; Bradler et al., 1991; Ben-Yoseph et al., 1993; Schurr et al., 1999), which are the gold standard for electrophysiological studies since slices preserve the anatomy of neuronal circuits and synaptic properties of excitability and plasticity (Bahr et al., 1995). However, the physiological performance of hippocampal slices ultimately depends on its metabolic dynamics, which has been poorly studied. As would be expected from its ability to endure prolonged periods of electrical activity, it has already been shown that hippocampal slices are metabolically competent (e.g. Whittingham et al., 1984; Bradler et al., 1991; Schurr et al., 1999), but no detailed characterization of the intermediary metabolism of this preparation has yet been carried out. Therefore, to set the basis for future parallel electrophysiological and metabolic studies, the present work intends to characterize the metabolic profile of the superfused hippocampal slice preparation by ^{13}C-NMR isotopomer analysis after labeling with [U-^{13}C]glucose and [2-^{13}C]acetate, to evaluate both neuronal and astrocytic metabolic compartments (Cerdán et al., 1990; Melo et al., 2006).

EXPERIMENTAL PROCEDURES

Reagents

[U-^{13}C]glucose (99%), sodium [2-^{13}C]acetate (99%) and $^{2}H_2O$ (99.9%) were purchased from Isotec Inc. (Miamisburg, OH, USA). 4-Aminopyridine (4AP) was purchased from Alomone Laboratories (Jerusalem, Israel). Solutions of ^{2}HCl (20% w/w) and NaO^{2}H (40% w/w) and other common reagents (highest purity available) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Carbogen (gas mixture of 95% O_2+5 % CO_2) was purchased to Linde Sogás (Lisbon, Portugal).

Preparation and superfusion of hippocampal slices

All experiments were conducted according to EU guidelines on ethical use of experimental animals (86/609/EEC), with particular care to minimize both animal suffering and the number of animals

*Corresponding author. Tel: +351-239-820190; fax: +351-239-822776.
E-mail address: racunha@clix.pt (R. A. Cunha).

Abbreviations: ACS, acyl-CoA synthetase; Cre, creatine; LDH, lactate dehydrogenase (E.C. 1.1.1.27); NMR, nuclear magnetic resonance; PC, pyruvate carboxylase (E.C. 6.4.1.1); PCA, perchloric acid; PCre, phosphocreatine; PDH, pyruvate dehydrogenase (E.C. 1.2.4.1); TCA, tricarboxylic acid; Y, anaplerotic flux; 4AP, 4-aminopyridine.

0306-4522/07$30.00+0.00
doi:10.1016/j.neuroscience.2006.11.027

used in each experiment. Male Wistar rats (8 weeks old) were anesthetized with halothane and decapitated. The brain was rapidly removed and the isolated hippocampi were transversely cut in 400 μm slices using a McIlwain tissue chopper. Hippocampal slices were allowed to recover in a modified Krebs solution (in mM: 115 NaCl, 25 $NaHCO_3$, 3 KCl, 1.2 KH_2PO_4, 2 $CaCl_2$, 1.2 $MgSO_4$, 5.5 glucose and 2 sodium acetate, previously and continuously gassed with carbogen, pH 7.4), at room temperature and during 45 min. Hippocampal slices were transferred to a submerged chamber and superfused (3 mL/min) with the same gassed solution at 37 °C. After 60 min to allow stabilization, the unlabeled glucose and acetate from the superfusion solution were substituted by [U-^{13}C]glucose and [2-^{13}C]acetate, respectively. The superfusion with these ^{13}C-tracers was performed for periods up to 7.5 h. In a set of experiments, slices were superfused for 3 h with 50 μM 4AP to trigger intermittent burst-like stimulation (Tibbs et al., 1989).

Metabolite extraction

Hippocampal slices were transferred to liquid nitrogen after each superfusion protocol, and the water-soluble metabolites were extracted with 7% (v/v) perchloric acid (PCA). The ground tissue mixed with PCA (300 μL/hippocampus) was centrifuged at 21,000×*g* during 15 min, at 4 °C. The supernatant was neutralized with KOH and lyophilized.

NMR spectroscopy

The lyophilized extracts were re-dissolved in 600 μL ^2H_2O and the p^{2}H was re-adjusted to 7.0 with ^{2}HCl or NaO^{2}H solutions. Sodium fumarate (2 mM) was used as an internal standard for quantification by ^{1}H-NMR spectroscopy. NMR spectra were acquired at 25 °C on an 11.7 T Varian Unity-500 spectrometer (Varian, Palo Alto, CA, USA), using a 5 mm broadband probe. Solvent-suppressed ^{1}H-NMR spectra were acquired with 8 kHz sweep width, using 14 s delay for allowing total proton relaxation, 3 s water pre-saturation, 70° pulse angle, 3 s acquisition time, and at least 200 scans per tissue extract (or 10 scans for perfusate samples). Proton decoupled ^{13}C-NMR spectra were acquired using 30 kHz sweep width, 45° pulse angle, 1.5 s acquisition time and a 1.5 s relaxation delay. The total repetition time (3 s) allowed full relaxation of the aliphatic carbons. To achieve adequate signal to noise ratio, the number of scans recorded was at least 10,000. Proton decoupled ^{31}P-NMR spectra were acquired with a 32 kHz sweep width, 45° pulse angle and a 2.2 s acquisition time, followed by 3 s delay. For each ^{31}P spectrum at least 6000 scans were acquired. In both ^{13}C- and ^{31}P-NMR spectra, ^{1}H broadband decoupling was achieved using a WALTZ-16 decoupling sequence.

All the acquired spectra were processed using the NUTS^{TM} software (Acorn NMR, Fremont, CA, USA). Free induction decays were baseline corrected, zero-filled and multiplied by an exponential function (0.5 Hz for ^{1}H and ^{13}C spectra; 10 Hz for ^{31}P spectra), prior to Fourier transformation. The areas of relevant signals in the spectra were quantified by curve fitting. The concentrations of metabolites, assigned according to Govindaroju et al. (2000), were determined by measuring the peak areas in the ^{1}H-NMR spectra, using sodium fumarate as internal standard, and normalized to total creatine (Cre) content. The ^{13}C isotopomer populations were determined from the analysis of ^{13}C-NMR spectra by deconvolution of the multiplets present in the resonance of each carbon, and each multiplet area was reported as a fraction of the total area for that specific carbon resonance. Phosphocreatine (PCre)/ATP and ATP/ADP ratios were calculated from the ^{31}P-NMR spectra, averaging the areas of the three ATP and the two ADP resonances.

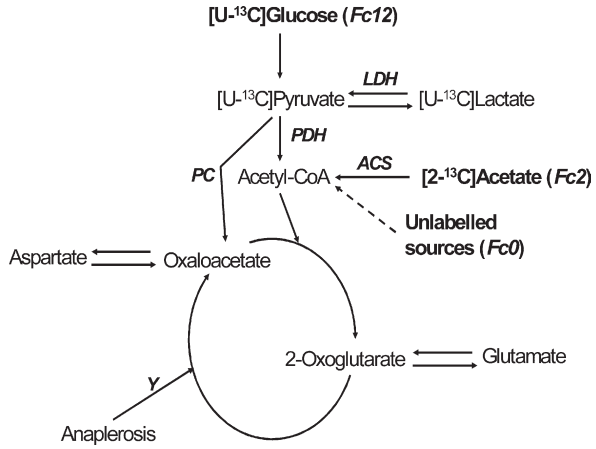

Fig. 1. Metabolic model used to fit the ^{13}C isotopomer data in the tcaCALC program. Fc0 corresponds to the contribution of unlabeled acetyl-CoA from unlabeled endogenous substrates; Fc2 and Fc12 show the fraction of acetyl-CoA labeled in C2 (originated from [2-^{13}C]acetate) and both C1 and C2 (originated from [U-^{13}C]glucose), respectively. Fluxes depicted in this model: see text for flux description.

Metabolic modeling

Multiplets from glutamate (C2, C3, C4), GABA (C2, C3) and aspartate (C2, C3) resonances in the ^{13}C-NMR spectra were used in the estimation of relative fluxes feeding the tricarboxylic acid (TCA) cycle using tcaCALC (Malloy et al., 1988; Fonseca et al., 2005). Glutamate and GABA isotopomer data were used in separated fittings and in combination with aspartate isotopomer data. The model used to fit the ^{13}C data are presented in Fig. 1 and includes the following parameters: fraction of acetyl-CoA derived from [2-^{13}C]acetate (Fc2); fraction of acetyl-CoA derived from the oxidation of [U-^{13}C]glucose (Fc12) via pyruvate dehydrogenase (PDH); fraction of acetyl-CoA formed from unlabeled precursors (Fc0) through acyl-CoA synthetase (ACS); combined anaplerotic flux from all sources (Y); flux through pyruvate carboxylase (PC); flux through lactate dehydrogenase (LDH). The estimated flux parameters are relative to the TCA cycle flux, arbitrarily set to 1.

Statistical analysis

All results are presented as mean±S.E.M. Student's unpaired two-tailed *t*-test or one-tailed ANOVA followed by the Newman-Keuls multiple comparison test was used to compare experimental data, and differences were considered significant when $P<0.05$.

RESULTS

Metabolic status and stability over time of superfused hippocampal slices

Fig. 2 shows typical proton spectra from perchloric extracts of hippocampal slices superfused in the presence of unlabeled acetate and glucose for a period of 3 h in the absence (A) and in the presence (B) of 4AP (50 μM) to trigger intermittent burst-like neuronal activity. The stimulation of the slices with 4AP induced a different metabolic status, namely an increase in amino acids such as glutamate, aspartate and GABA, which are quantified in Table 1. The profile in the proton spectra recorded after 3 h of superfusion in the presence of unlabeled acetate and glucose was maintained from 1.5 up to 7.5 h (spectra not