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Na^+/Cl^-/CREATINE TRANSPORTER ACTIVITY AND EXPRESSION IN RAT BRAIN SYNAPTOSOMES

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Abstract—Creatine is involved in brain ATP homeostasis and it may also act as neurotransmitter. Creatine transport was measured in synaptosomes obtained from the diencephalon and telencephalon of suckling and 2 month-old rats. Synaptosomes accumulate [^{14}C]-creatine and this accumulation was Na^+- and Cl^--dependent and inhibited by high external K^+. The latter suggests that the uptake process is electrogenic. The kinetic study revealed a K_m for creatine of 8.7 μM. A 100-fold excess of either non-labelled creatine or guanidinopropionic acid abolished NaCl/creatine uptake, whereas GABA uptake was minimally modified, indicating a high substrate specificity of the creatine transporter. The levels of NaCl/creatine transporter (CRT) activity and those of the 4.2 kb CRT transcript (Northern's) were higher in the diencephalon than in the telencephalon, whereas the 2.7 kb transcript levels were similar in both brain regions and lower than those of the 4.2 kb. These observations suggest that the 4.2 kb transcript may code for the functional CRT. CRT activity and mRNA levels were similar in suckling and adult rats. To our knowledge the current results constitute the first description of the presence of a functional CRT in the axon terminal membrane that may serve to recapture the creatine released during the synapsis.

Key words: creatine transport, CRT, brain.

Until recently the only known role for creatine in brain was that played in ATP homeostasis via the creatine/phosphocreatine/creatine kinase system (Brosnan and Brosnan, 2007; Andres et al., 2008). Almeida et al. (2006) reported that neurons release creatine in an action potential-dependent manner, suggesting a role for creatine as a neurotransmitter, particularly as co-transmitter on GABA postsynaptic receptors. It has also been suggested that creatine regulates appetite and weight by acting on specific hypothalamic nuclei (Galbraith et al., 2006).

The physiological relevance of creatine is strengthened by the devastating consequences that result from creatine deficiency. Three disorders that cause either absence or a severe decrease of creatine in the brain have been described: guanidinoacetate-methyltransferase (GAMT), arginine: glycine amidinotransferase (AGAT) and Na^+/Cl^-/creatine transporter (SLC6A8 or CRT) deficiencies (see reviews of Brosnan and Brosnan, 2007; Andres et al., 2008). In AGAT- and GAMT-deficient patients, high doses of oral creatine supplementation produces a partial restoration of the cerebral creatine pool after several months. The liver and kidney synthesize most of the body's creatine, but AGAT and GAMT mRNAs have been detected in the embryonic and adult rat brain, suggesting that the brain may synthesis its own creatine. However, despite the presence in brain of the creatine biosynthetic enzymes, the symptoms due to CRT deficiency cannot be corrected by creatine supplementation (see review of Brosnan and Brosnan, 2007; Andres et al., 2008).

The creatine concentration in the mammalian brain is 4 to 20-fold greater than that in the liver and possibly the major supply route of creatine to the brain is of peripheral origin. CRT mRNA has been identified throughout the rat brain (Schloss et al., 1994; Happe and Murrin, 1995; Saltarelli et al., 1996; Braissant et al., 2001, 2005; Galbraith et al., 2006) with high levels in the myelinated tracts, cerebellar granule cells, hippocampal pyramidal cells, several brainstem nuclei and the choroid plexus (Saltarelli et al., 1996). Within the brain cells, CRT mRNA was detected in neurons and oligodendrocytes but not in astrocytes (Braissant et al., 2001, 2005; Ohtsuki et al., 2002).

As Na^+/Cl^-/creatine transport (SLC6A8 or CRT) activity has only been measured at the plasma membrane (see reviews of Speer et al., 2004; Brosnan and Brosnan, 2007), the presence of CRT mRNA in neurons suggests the presence of CRT protein in their plasma membrane. The mitochondria also transports creatine (Wazel et al., 2002). However, CRT does not appear to mediate creatine transport in the mitochondria because the mitochondria creatine transporter has different sensitivity to creatine analogs and a 1000 times lower affinity for creatine than the plasma membrane CRT. In addition, mitochodria creatine transport must be independent of the electrochemical Na^+ gradient.

Creatine uptake activity has been observed in rat cortical cultures (Almeida et al., 2006), neuroblastoma cell cultures (Daly, 1985), astroglial-rich cell cultures (Möller and Hamprecht, 1989) and hippocampal slices (Lunardi et al., 2006). These studies, however, do not characterized brain creatine transport in terms of affinity and specificity for creatine, and of ionic and membrane voltage dependence. The present work shows that synaptosomes obtained from rat diencephalon and telencephalon have in their membranes a functional Na^+/Cl^-/creatine transporter (CRT).

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Abbreviations: AGAT, arginine: glycine amidinotransferase; CRT, Na^+/Cl^-/creatine transporter; GAA, guanidinoacetic acid; GAMT, guanidinoacetate-methyltransferase; GPA, guanidinopropionic acid.

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EXPERIMENTAL PROCEDURES

Chemicals

[^{3}H]-GABA and [α^{32}P]-UTP were purchased from GE Healthcare, Europe, GmbH, [^{14}C]-creatine from Moravek Biochemicals Inc., USA and anti-CRT antibody from Alpha Diagnostic International, Inc., USA. All the other reagents used were obtained from Sigma-Aldrich, Spain.

Animals

Eighteen days-old (suckling) and 2 month-old (adults) Wistar rats were used in the current study because in the kidney the maximal differences in CRT activity versus age were found between suckling and 2 month-old rat (García-Delgado et al., 2007). They were provided by the animal care facility of the University of Sevilla. Rats were humanely handled and sacrificed in accordance with the European Council legislation 86/609/EEC concerning the protection of experimental animals. Mothers and adult rats were fed with a rat chow diet (Panlab 04) *ad libitum* and had free access to water.

Solutions and synaptosomes preparation

The brains of three to four suckling rats and of two adult rats were pooled to obtain a synaptosomal preparation of either telencephalon or diencephalon. Rats were anesthetized with a lethal i.p. injection of pentobarbital (50 mg/kg) and the brain was rapidly removed and dropped into ice cold PBS solution. The telencephalon and diencephalon were dissected according to the atlas of Paxinos and Watson (1986), cutting off the olfactory bulb, striatum nucleus and hippocampus.

Synaptosomal fractions from telencephalon and diencephalon were prepared using a discontinuous ficoll gradient as described by Booth and Clark (1978) with some modifications. Briefly, the tissues were homogenized in (in mmol/L) 320 sucrose, 1 EDTA, 1 EGTA, 10 Tris–HCl (pH 7.4). The homogenate was spun at 1000 g for 10 min and the resultant supernatant was centrifuged at 12,000 g for 20 min. The pellet was re-suspended in 3 ml of (in mmol/L) 320 sucrose and 10 Tris–HCl (pH 7.4). The 3 ml were carefully loaded in a discontinuous ficoll gradient (13% and 7.5% ficoll (w/v) in 320 mmol/L sucrose and 10 mmol/L Tris–HCl, pH 7.4). The gradient was centrifuged at 98,000 g for 30 min in a swing-out rotor centrifuge. Myelin and synaptosomes banded at the first and second interphases respectively, with the mitochondria being pelleted at the bottom. The synaptosomal band was collected and spun at 12,000 g for 20 min. The final synaptosomal pellet was re-suspended in a pH 7.4 buffer containing (in mmol/L) 240 mannitol, 10 glucose, 4.8 Kgluconate, 2.2 Cagluconate, 1.2 $MgSO_4$, 1.2 KH_2PO_4 and 25 HEPES-Tris. All of the steps were carried out at 4 °C.

Protein and enzyme assays

Protein was measured by the method of Bradford (1976), using γ globulin as the standard.

Enzyme assays were carried out in a Hitachi U-2800A recording spectrophotometer. Citrate synthase activity was assayed at 25 °C by the method of Srere (1969), in the presence of 0.1% TritonX-100 to release maximal activity. Ouabain-sensitive, K^+-activated phosphatase activity, a partial reaction of the Na^+, K^+, ATPase, was assayed at 37 °C by the method of Colas and Maroux (1980), in the presence of 20 mM K^+, using p-nitrophenyl phosphate as a substrate. Ouabain-sensitive, K^+-activated phosphatase was defined as the difference in activity measured in the presence and absence of 2 mM ouabain.

Creatine and GABA uptake into synaptosomes

Either [^{14}C]-creatine or [^{3}H]-GABA uptake into synaptosomes was measured at 37 °C using a rapid filtration technique as described (García-Delgado et al., 2007). Briefly, 200–250 μg of synaptosomes were added to tubes containing (in mmol/L) 100 NaCl, 40 mannitol, 10 glucose, 4.8 Kgluconate, 2.2 Cagluconate, 1.2 $MgSO_4$, 1.2 KH_2PO4, 25 HEPES-Tris (pH 7.4) and either 6 μM [^{14}C]-creatine or 10 nM [^{3}H]-GABA. When required NaCl was isosmotically replaced by mannitol. After designated periods of time, uptake was terminated by the addition of 1 ml of NaCl-free ice cold buffer (stop solution). The samples were immediately filtered, under vacuum, through a 0.45 μm pore size Millipore filter pre-wetted with water. Filters were further washed with 5 ml of ice cold stop solution, dissolved in 5 ml of Ready-Protein (Beckman, Germany) scintillation fluid and the radioactivity determined by liquid scintillation spectrometry. Non-specific isotope binding to the filters was determined separately by adding stop solution to the synaptosomes before addition of uptake buffer and it was subtracted from the total radioactivity of each sample. All experiments were done in triplicate.

RNA preparation and Northern blotting

Total RNA was extracted from either telencephalon or diencephalon as described by Chomczynski and Sacchi (1987) and Poly (A^+)RNA was isolated by the method of Badley et al. (1988) as previously reported by us (Murillo-Carretero et al., 1999). [α^{32}P]-UTP-labelled antisense riboprobe of CRT was generated from a rat cDNA fragment as described (Peral et al., 2002). 10 μg of poly(A^+) RNA were loaded into individual lanes of a single gel. Relative quantification of mRNA was determined with a phosphoimager system (Fuji Photo Film Co., Ltd.) using the PCBAS program (Raytest GmbH). Expression of CRT mRNA was normalized to levels of cyclophilin mRNA. The size of the transcripts was evaluated by ribosomal RNA.

RT-PCR assays

Total RNA was extracted from either telencephalon or diencephalon using RNeasy® kit (Qiagen, USA). cDNA was synthesized from 1 μg of total RNA using QuantiTest® reverse transcription kit (Qiagen) as described by the manufacturer. Real-time PCR was performed with iQ™SYBR® Green Supermix (BioRad, Spain), 0.4 μM primers and 1 μl cDNA, as described (García-Delgado et al., 2007). Controls were carried out without cDNA. Amplification was run in a Mini-iOpticon™ System (BioRad) thermal cycler (94 °C/3 min; 35 cycles of 94 °C/40 s; 58 °C/40 s, and 72 °C/40 s). Following amplification, a melting curve analysis was performed by heating the reactions from 65 to 95 °C in 1 °C intervals while monitoring fluorescence. Analysis confirmed a single PCR product at the predicted melting temperature. Primers for CRT were based upon the published sequence of rat CRT (GenBank Database) and were antisense, TTCTATTACCTGGTCAAGTCCT and sense, GCCTCAAGACTTTGTTCTCC. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as reference gene and was used for samples normalization. The primer sequences for GAPDH were antisense, TGCACCACCAACTGCTTAGC and sense, GGCATGGACTGTGGTCATGAG. The cycle at which each sample crossed a fluorescence threshold, Ct, was determined, and the triplicate values for each cDNA were averaged. Analyses of real-time PCR were done using the comparative Ct method with the Gene Expression Macro software supplied by BioRad.

Western assays

SDS-Page was performed according to Laemmli (1970) on a 10% polyacrylamide gel. Protein samples of synaptosomes obtained from diencephalon and telencephalon of suckling and 2 month-old