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Pharmacological characterization of glutamate Na^+-independent transport in retinal cell cultures: Implications in the glutathione metabolism

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ABSTRACT

L-Glutamate is the primary excitatory neurotransmitter in the mammalian central nervous system (CNS). Mechanisms for the removal of glutamate are vital for maintaining normal function of retina. In the present study, using retinal cell cultures obtained from chick embryos, we characterize, pharmacologically, the presence of two glutamate transporter mechanisms, Na^+-dependent and Na^+-independent uptake systems. Na^+-independent uptake system seems to present characteristics related to system x_{CG}^- (cystine–glutamate exchanger) that in the current work demonstrated highlighted contribution to the glutamate transport in retina, which is not observed in other tissues. Our results showed that glutamate shares x_{CG}^- system with another amino acid, L-cysteine, suggesting the possible involvement of this component in processes related to the release of the glutathione antioxidant molecule. Furthermore, cysteine uptake by Na^+-independent transport appears to be more evident in glial cell cultures than in neuronal cell cultures. So, Na^+-independent transport system seems to have other functions besides amino acid transport, demonstrating a physiological role in modulating cell redox status.

L-Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system (CNS), and has been involved in many complex physiological processes such as developmental plasticity, memory, learning and long-term potentiation (Quinlan et al., 1999; Shigeri et al., 2004). In the retina, this amino acid is responsible for the synaptic transmission between photoreceptor cells, bipolar cells and ganglion cells and its excessive action in different types of receptors may result in neuronal cell death by excitotoxicity (Kalloniatis and Napper, 1996; Rauen et al., 1996).

Several studies have been demonstrated the potential involvement of glutamate-mediated toxicity in both acute (traumatic brain injury, ischaemia) and chronic (Alzheimer's disease, Huntington's disease, glaucoma and most recently, Wernicke's encephalopathy) neurodegenerative conditions (Suemori et al., 2006; Sheldon and Robinson, 2007; Hazell, 2009).

Thereby, termination of glutamatergic neurotransmission is achieved by the removal of glutamate from the extracellular space by molecular transporters located in the plasmatic membrane of pre-synaptic terminals and surrounding glial cells (O'Shea, 2002; Beart and O'Shea, 2007). In the CNS, excitatory amino acid transporters (EAATs) are the main responsible for the removal of glutamate neurotransmitter, maintaining its extracellular concentration below excitotoxic levels (Danbolt, 2001; Balcar, 2002; Kanai and Hediger, 2004). The transport of glutamate by EAATs is characterized as electrogenic, once it is thermodynamically coupled to the cotransport of at least two sodium ions (Na^+), one proton (H^+) and the counter-transport of a potassium ion (Tanaka, 2000; Amara and Fontana, 2002; Bridges and Esslinger, 2005). Thereby, these transporters are known as Na^+-dependent high-affinity glutamate transporters (Shigeri et al., 2004; Bridges and Esslinger, 2005). This transport is, also, coupled to a Cl^- channel, which function has not already been described.

Furthermore, a great number of different membrane transporters have already been characterized, regulating the flux of glutamate in distinct areas of the CNS, including the retina. These systems are commonly differentiated based upon their ionic dependence and include both sodium-independent system x_{CG}^- (Baker et al., 2002; Patel et al., 2004) and sodium-dependent system x_{AG}^- represented by the EAATs (Palacin et al., 1998; McBean, 2002).

System x_{CG}^- (cystine-glutamate exchanger) is a member of the glycoprotein-associated amino acid transporter family and has already been described in hepatocytes, alveolar type II cells, human

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endothelial cells and macrophages (Ishii et al., 1992). In the CNS, this system was identified in primary cultures of neurons (Sagara et al., 1993) and astrocytes (Allen et al., 2001; Gochenauer and Robinson, 2001); C6 glioma cells (Cho and Bannai, 1990); microglia (Piani and Fontana, 1994) and human gliomas (Ye et al., 1999).

Under physiological conditions, system x_{CG}^- transports cystine into cells coupled to the efflux of intracellular glutamate, by a Na^+-independent mechanism (Bender et al., 2000). Once taken up by cells, cystine is rapidly and spontaneously reduced to cysteine, which is required for the synthesis of glutathione (GSH), an endogenous antioxidant essential for cellular defense (McBean, 2002; Tomi et al., 2003). The tripeptide GSH is produced from the amino acids glutamate, cysteine and glycine by the consecutive actions of two enzymatic reactions (Yoneyama et al., 2008) and its role as a free radical scavenger is particularly important in the retina since this tissue is extremely vulnerable to oxidation because of its high oxygen consumption, high unsaturated fatty acid content and exposure to light (Handelman and Dratz, 1986; Ahuja et al., 2005). Furthermore, system x_{CG}^- can also mediate the influx of glutamate when its concentration outside the cell is higher (Bringmann et al., 2009).

Indeed, it is becoming increasingly relevant the importance of the inter-dependency between neurons and astrocytes in maintaining the level of some intracellular molecules and amino acids (Struzynska, 2009). Cysteine, the rate-limiting substrate for the glutathione synthesis, is one of these amino acids, which is continuously released from astrocyte and taken up by neuronal cells (Wang and Cynader, 2000; Stewart et al., 2002; Bringmann et al., 2009).

Both *in vivo* and *in vitro* studies demonstrated that GSH levels are, usually, higher in astrocytes than in neurons, varying in function of the CNS area (Langeveld et al., 1996; Cooper and Kristal, 1997; Dringen et al., 2000; Struzynska, 2009). In culture models, the low levels of GSH in neurons are justified by the fact that these cells are unable to transport cystine, rely on the availability of cysteine for their GSH synthesis (Kranich et al., 1996).

In C6 glioma cells, cystine used for GSH synthesis is obtained, principally, by the transport system x_{CG}^- (Cho and Bannai, 1990). Although, it is not clear which system, x_{AG}^- or x_{CG}^-, is the principal responsible for the levels of GSH and also, the mechanisms used by systems during this process.

Besides, a wide variety of works are being developed, trying to elucidate the mechanisms whereby GSH can be released by cells (Dringen et al., 1997, 1999). Sagara et al. (1996) suggested that, in astrocytes, this release could be ion independent and mediated by a carrier protein, although little is known about this process. Recently, the multidrug transporter protein (MRP1) has been shown to be responsible for the efflux of GSH from astrocytes under oxidative stress conditions, although it is not clear the involvement of this carrier under physiological conditions (Hirrlinger et al., 2001; Minich et al., 2006).

Based on that, in the present work, primary retinal cell cultures were employed as an experimental model to assess the participation of Na^+-dependent and Na^+-independent systems in the glutamate and cysteine transport, highlighting the possible involvement of these systems in the metabolism of glutathione.

1. Materials and methods

1.1. Materials

Cell culture reagents were obtained from GIBCO (Paisley, UK) and tissue culture plasticware from TPP. L-[^{3}H]Glutamate (specific activity—1050 Ci/mmol) and L-[^{35}S]Cysteine (specific activity—1050 Ci/mmol) were purchased from Amersham Bioscience Biotech (Hampshire, UK). Polyornithine, L-glutamate, cysteine, glutathione and transporter inhibitors, such as DL-*threo*-β-benzyloxyaspartate (TBOA) and L-*trans*-pyrrolidine-2,4-dicarboxylic acid (PDC) were provided by Sigma–Aldrich (St. Louis, MO, USA). All other reagents were of analytical grade. Fertilized white Leghorn eggs were obtained from a local hatchery and maintained at a humid atmosphere.

1.2. Retinal cell culture

Chick embryos were staged according to Hamburguer and Hamilton (1951) and neuron/glia-mixed cultures were prepared as previously described by Herculano et al. (2006). Briefly, under sterile conditions, 7–8-day old chick embryo (E7–E8) retinas were properly dissected on calcium and magnesium-free salt balanced solution (CMF) at 0–4 °C, cleared from the pigmented epithelium and other ocular tissues. For enzymatic dissociation, retina was transferred to a glass containing trypsin (0.05%) for 3–5 min at 37 °C. After, the tissue was mechanically dissociated with a pipette and then submitted to a brief centrifugation. The pellet was suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and 500 μg/ml glutamine. Cells were seeded in 35 mm plastic dishes, pretreated with polyornithine (10 μg/ml during 30 min) and maintained at 37 °C in a humidified 5% CO_2—95% air atmosphere. After 7–8 days *in vitro* (DIV7-DIV8), cultures showed a confluent monolayer containing both neurons and glial cells at a density of 2–3 × 10^6 cells per plate.

1.3. Retinal glial cell culture

Purified glial cell cultures were prepared from the retina of 9–10-day old chick embryos as described earlier. After carefully dissecting, retinal cells were plated into 35 mm cultures dishes without pretreatment with polyornithine. The cultures were maintained in DMEM with 10% FBS during, at least, 15 days (DIV15) until confluent monolayer was formed, containing, predominantly, glial cells (≅95%) and a small number of neurons (≅5%). In order to accelerate neuronal cell death, the culture medium was replenished every 3 days.

1.4. Retinal neuronal cell culture

Retinal neuronal cell cultures were also prepared from 7-day old chick embryos using the same procedures above, with slight modifications. Cells were cultivated in DMEM supplemented with 1% FBS in 35 mm plates and pretreated with polyornithine. Neuronal cultures were maintained during 5 days *in vitro* (DIV5) at 37 °C in an atmosphere containing 95% of air and 5% of CO_2, and after this period no glial contamination (less then 5%) was detected in confluent neuronal culture.

1.5. L-[^{3}H]Glutamate and L-[^{35}S]Cysteine uptake assay in primary retinal cell cultures

After reaching full confluency, cultures were used to perform the uptake assay as described previously (do Nascimento et al., 1998). To evaluate glutamate uptake, retinal cultures were washed four times with modified Hank's balanced salt solution, which consists of (in mM): NaCl 128, KCl 4, $MgCl_2$ 1, $CaCl_2$ 2, glucose 12 and HEPES 20, adjusted to pH 7.4, and then incubated with [^{3}H]Glutamate (1 μCi/ml) during 5 min. To study Na^+-dependent glutamate transport, sodium chloride (NaCl) was replaced by lithium chloride (LiCl) in the uptake buffer. All uptake assays were performed at room temperature (37 °C). After [^{3}H]Glutamate time incubation, the uptake was stopped by rapidly rinsing the cells three times with ice-cold Hank's solution and then, cells were lysed with 5% TCA. The radioactivity presented in the cells was determined by liquid scintillation spectroscopy.

To further characterize the effect of glutamate transport inhibitors, cells were pretreated (before [^{3}H]Glutamate incubation) during 10 min with TBOA and PDC, separately, at 10 μM, 50 μM, 100 μM and 500 μM. Indeed, the same procedure was used to evaluate the effect of cysteine.

L-[^{35}S]Cysteine uptake assay was assessed according to the methodology described above, substituting [^{3}H]Glutamate by [^{35}S]Cysteine.

Likewise, the same procedure was applied to evaluate glutamate and cysteine transport in the three types of cultures used in the present work.

1.6. Glutathione assay

Intracellular and extracellular levels of glutathione (GSH) were determined by the method described by Anderson (1969) with minor modifications. This procedure allows to determine, spectrophotometrically, the total levels of glutathione (GSH and GSSG) by the reduction of 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) in nitrobenzoic acid (TNB). In this reaction, the sample is incubated in two different groups: in the first group the sample is incubated only with DTNB to measure the levels of –SH group and in the second group the sample is incubated with GSH reductase (that converts GSSG into GSH), NADPH and DTNB to measure the total levels of GSH produced by the reaction. Then, the final result is expressed by the difference between these two groups as the total levels of glutathione.

To evaluate the relationship between Na^+-independent transport and GSH content, the culture medium was removed and the cells were quickly washed three times with Hank's solution (pH 7.4). Subsequently, cultures were incubated, during 90 min with: Hank's solution—LiCl (as control); cysteine (500 μM); PDC (500 μM) and cysteine (500 μM) + PDC (500 μM).

After the treatment, the incubation medium was removed and substituted with PBS/EDTA (1 mM) to scrape the cells. An aliquot was separated to protein determination, using the method described by Lowry et al. (1951). The left volume was centrifuged at 3000 rpm, during 10 min.