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Gap junction hemichannel-mediated release of glutathione from cultured rat astrocytes

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Abstract

Release of glutathione (GSH) from astrocytes is essential for the supply of neurons with the GSH precursor cysteine. In order to test whether gap junction hemichannels could contribute to GSH release from astrocytes, we incubated astrocyte-rich primary cultures from neonatal rat brain in the absence of divalent cations, a condition that is known to increase the opening probability of hemichannels. During incubation in divalent cation free incubation solution (DCFS) the cells remained viable and released about 50% of the initial cellular GSH within 15 min. This extracellular GSH accumulation in DCFS was lowered by the presence of Ca^{2+} in a concentration dependent manner with a half-maximal inhibition at a Ca^{2+} concentration of 107 ± 46 μM. Extracellular GSH accumulation in DCFS was also blocked by the divalent cations Mg^{2+}, Ba^{2+} and Sr^{2+} as well as by the known gap junction inhibitors carbenoxolone (CBX), flufenamic acid (FFA) and lanthanum chloride. In contrast, the $P2X_7$ receptor blocker brilliant blue G (BBG) did not affect GSH release in divalent cation free solution. This pharmacological profile strongly suggests that astrocytes are able to release GSH via open hemichannels. This release of GSH may have severe consequences for the antioxidative defense and for the GSH homeostasis in pathological brain.

Keywords: Astrocyte–neuron interactions; Gap junctions; Glutathione; Hemichannels; Transport

The tripeptide glutathione (GSH) is very important for the antioxidative defense of the brain. It reacts non-enzymatically with radicals and is the electron donor for the reduction of peroxides by glutathione peroxidases [3,4]. Astrocytes play a crucial role in the GSH metabolism of the brain, since these cells supply neighboring neurons with the precursors for GSH synthesis. The release of GSH from astrocytes is the essential first step in this metabolic interaction and is predominately mediated by the multidrug resistance protein 1 [13,18]. Extracellular GSH is processed by ectoenzymes to generate extracellular cysteine, the availability of which limits GSH synthesis in neurons [7].

Gap junctions are intercellular channels that control the diffusion driven passage of ions and molecules of a molecular mass of up to 1 kDa between cells [21,24]. Gap junctions are made of two hemichannels from two neighboring cells across the extracellular gap. Each hemichannel or connexons is built from six connexin proteins. In addition to their presence in gap junctions, stand alone hemichannels can also be present in cells. These hemichannels have a low opening probability that is increased under various physiological and pathological conditions [20,21].

Astrocytes express several isoforms of connexins that build up interastrocytic gap junctions as well as gap junctions to oligodendrocytes [24]. Also the presence of gap junction hemichannels has been demonstrated for cultured astrocytes by immunocytochemical staining of connexin protein as well as by the loading of the cells with lucifer yellow or ethidium bromide under conditions that increase the opening probability of hemichannels such as absence of divalent cations or metabolic inhibition [2,27].

Cultured astrocytes have been reported to release glutamate and ATP in divalent cation free (DCF) media via gap junction hemichannels [22,27]. Since the tripeptide GSH is sufficiently small to be transported through hemichannels, we used astrocyte-rich primary cultures as model system to test whether astrocytes may show increased GSH release in DCF solution. Astrocyte cultures were prepared from the brains of newborn

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Wistar rats as described previously in wells of 24-well plates [10,13] and used at a culture age between 15 and 28 days. The cultures were washed with 2 mL of divalent cation free incubation solution (DCFS: 20 mM HEPES, 145 mM NaCl, 5.4 mM KCl, 0.8 mM Na_2HPO_4, 5 mM glucose, pH 7.4) and then incubated at 37 °C on a water bath with 500 μL of DCSF containing 100 μM of the γ-glutamyl transpeptidase inhibitor acivicin (to prevent extracellular GSH degradation [5]) without or with divalent cations and/or inhibitors in the concentrations indicated in the figures and the table. After the indicated incubation periods the contents of cellular and extracellular total glutathione (GSx = amount of GSH plus twice the amount of glutathione disulfide) was determined as described previously [13,18] using the colorimetric Tietze method. Since cultured astrocytes contain under unstressed conditions at best minute amounts of glutathione disulfide (<1% of GSx [13,18]), the GSx values determined for cultured astrocytes represent almost exclusively GSH. Cell viability was analysed by determining the extracellular activity of lactate dehydrogenase (LDH) in the cultures [6]. The LDH activity in the incubation buffer after incubation was compared to the LDH activity in lysates of untreated astrocyte cultures that were obtained by exposing cultures to 1% Triton X-100 for 30 min [6]. The protein content of the cell cultures was determined with the method of Lowry et al. [17] using bovine serum albumin as a standard.

Exposure of cultured astrocyte to DCFS in the presence of 1.8 mM $CaCl_2$ caused some extracellular accumulation of GSx within the first 5 min of incubation that was followed by a slow further accumulation during longer incubation. A quick increase in extracellular GSx that followed the washing period has previously been observed [13] and is most likely attributed to some stress applied during the washing procedure. After this initial increase in extracellular GSx in the presence of Ca^{2+} extracellular GSx accumulation was slow (Fig. 1) and continued with a rate that was similar to those previously reported for cultured rat astrocytes of about 3 nmol/(h mg) protein [5,13,18]. This extracellular GSx accumulation is likely to represent predominantly active GSH transport via Mrp1 which is responsible for 60% of GSH release from cultured rat and mouse astrocytes [13,18].

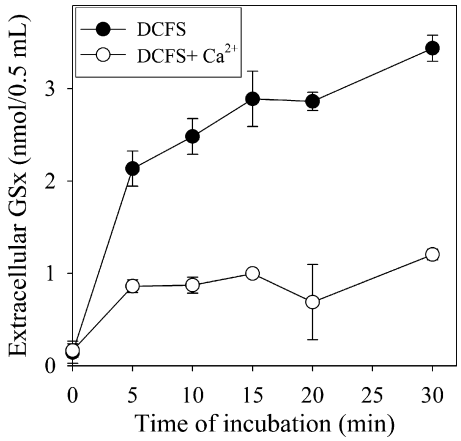

Fig. 1. Extracellular GSx accumulation during incubation of astrocytes in 500 μL DCFS without or with 1.8 mM $CaCl_2$. The cellular GSx content at the onset of the experiment was 34.3 ± 1.6 nmol/mg protein and the culture contained 104 ± 4 μg protein/well.

Incubation of cultured astrocytes in the absence of divalent cations resulted in a severe extracellular GSx accumulation that resulted within 15 min in extracellular GSx contents that were about thrice that found for cells incubated in the presence of Ca^{2+} (Fig. 1). The extracellular accumulation of GSx was rapid during the first 5 min of incubation and the extracellular GSx concentration did slowly increase further during longer incubation (Fig. 1). After 15 min incubation in DCFS the extracellular GSx content accounted for half of the total GSx content of the cultures, whereas in the presence of calcium 17% of the total GSx was found in the media and 83% was retained in the cells (Table 1). The increase in GSH accumulation in DCFS was not due to a loss in cell viability, since no significantly increased LDH release was observed compared to cells that had been incubated in the presence of Ca^{2+} (Table 1).

The strong extracellular GSx accumulation in the absence of divalent cations suggests that mechanisms become activated under such conditions which lead to a rapid loss of cellular GSH. Incubations of astrocytes in DCFS with the divalent cation chelator EDTA or EGTA in a concentration of 100 μM did not result in

Table 1
Consequences of a treatment of cultured astrocytes in DCFS in absence or presence of divalent cations or inhibitors

Compound	ΣGSx (nmol/mg)	Extracellular GSx (% of ΣGSx)	Cellular GSx (% of ΣGSx)	Extracellular LDH (% of total LDH)	*n*
None	25.5 ± 7.5	51 ± 11$^{###}$	49 ± 11$^{###}$	9.9 ± 2.5	25
Ca^{2+} (1.8 mM)	26.6 ± 7.0	17 ± 9***	83 ± 9***	6.5 ± 3.7	25
Mg^{2+} (1.8 mM)	26.1 ± 3.0	26 ± 7***	74 ± 8***	8.3 ± 3.4	9
Sr^{2+} (1.8 mM)	25.5 ± 5.2	20 ± 6***	80 ± 6***	14.5 ± 5.8	9
Ba^{2+} (1.8 mM)	23.8 ± 4.5	23 ± 7***	77 ± 7***	12.6 ± 6.0	9
FFA (100 μM)	25.4 ± 4.5	18 ± 5***	82 ± 5***	13.6 ± 6.0	12
CBX (10 μM)	27.9 ± 3.9	20 ± 6***	80 ± 6***	11.4 ± 5.5$^{##}$	12
$LaCl_3$ (100 μM)	28.5 ± 3.5	14 ± 7***	86 ± 7***	18.3 ± 13.7	12
BBG (1 μM)	23.9 ± 2.7	46 ± 12$^{###}$	54 ± 12$^{###}$	15.9 ± 10.7$^{##}$	12

Astrocyte cultures were incubated for 15 min in 500 μL DCFS without (none) or with the indicated compounds. Extracellular and cellular GSx contents as well as the extracellular LDH activity were determined. ΣGSx represent the specific GSx content of cells plus media after 15 min incubation. The data represent means ± S.D. of values obtained on *n* wells derived from two or more independently prepared astrocyte-rich primary cultures. The significance of differences obtained compared to DCFS treatment (none) ($^{***}p < 0.001$) or treatment with DCFS plus Ca^{2+} ($^{##}p < 0.01$; $^{###}p < 0.001$) was calculated using ANOVA followed by Bonferroni post hoc test.