Original Paper

Up-Regulation of the Excitatory Amino Acid Transporters EAAT1 and EAAT2 by Mammalian Target of Rapamycin

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Key Words

Neuroexcitability • Glutamate transporter • SLC1A3 • SLC1A2

Abstract

Background: The excitatory amino-acid transporters EAAT1 and EAAT2 clear glutamate from the synaptic cleft and thus terminate neuronal excitation. The carriers are subject to regulation by various kinases. The EAAT3 isoform is regulated by mammalian target of rapamycin (mTOR). The present study thus explored whether mTOR influences transport by EAAT1 and/or EAAT2.

Methods: cRNA encoding wild type EAAT1 (SLC1A3) or EAAT2 (SLC1A2) was injected into *Xenopus* oocytes without or with additional injection of cRNA encoding mTOR. Dual electrode voltage clamp was performed in order to determine electrogenic glutamate transport (I_{EAAT}). EAAT2 protein abundance was determined utilizing chemiluminescence. **Results:** Appreciable I_{EAAT} was observed in EAAT1 or EAAT2 expressing but not in water injected oocytes. I_{EAAT} was significantly increased by coexpression of mTOR. Coexpression of mTOR increased significantly the maximal I_{EAAT} in EAAT1 or EAAT2 expressing oocytes, without significantly modifying affinity of the carriers. Moreover, coexpression of mTOR increased significantly EAAT2 protein abundance in the cell membrane. **Conclusions:** The kinase mTOR up-regulates the excitatory amino acid transporters EAAT1 and EAAT2.

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Introduction

Excitatory amino acid transporters clear glutamate and aspartate from synaptic clefts and thus participate in the termination of neuronal excitation as well as protection against excitotoxicity [1-5]. The excitatory amino acid transporter isoforms EAAT1 and EAAT2 are

the most important carriers clearing glutamate and aspartate following excitation [6]. EAAT1 primarily mediates glutamate uptake into glial cells [1]. The carrier is, however, not only expressed in astrocytes [7-15] and oligodendrocytes [16, 17], but in addition in neurons [18-20], retina [21, 22], taste buds [23], cochlea [24, 25], vestibular organ [26], circumventricular organ [7], as well as adrenal and pineal glands [27, 28]. EAAT2 is expressed in astrocytes [29] and accomplishes glial uptake of excitatory amino acids from the synaptic cleft [30]. Upregulation of EAAT2 activity provides neuroprotection [31] and impaired expression or activity of EAAT2 leads to extracellular glutamate accumulation and neuroexcitotoxicity [32-35].

The EAAT3 excitatory amino acid transporter isoform is regulated by mammalian target of Rapamycin (mTOR) [36], a serine/threonine kinase stimulated by growth factors [37], insulin [38], leptin [39], and nutrients [40]. The kinase is down-regulated by hypoxia [41] and contributes to the regulation of cell proliferation, cell growth and cell survival [37, 40, 42].

The present study explored whether the glutamate transporters EAAT1 (SLC1A3) or EAAT2 (SLC1A2) are similarly sensitive to mTOR. To this end, EAAT1 or EAAT2 were expressed in *Xenopus* oocytes without and with additional expression of mTOR. Glutamate transport was quantified by measurement of glutamate-induced current, as determined by dual electrode voltage clamp.

Materials and Methods

Constructs

For generation of cRNA [43, 44], constructs were used encoding wild-type mTOR [45], EAAT1 [46] and EAAT2 [47-49]. The constructs were sequenced to verify the correctness of the plasmid [50]. The constructs were used for generation of cRNA as described previously [51-53].

Voltage clamp in *Xenopus* oocytes

Xenopus oocytes were prepared as previously described [54-56]. 10 ng of wild type mTOR cRNA were injected one day after preparation of the oocytes and 10 ng EAAT1 or EAAT2 cRNA was injected one day after mTOR injection. The oocytes were maintained at 17°C in a solution containing (in mM: 96 NaCl, 4 KCl, 1.8 MgCl₂, 0.1 CaCl₂, 5 HEPES, pH 7.4, supplemented with theophyllin (90 mg/l), gentamycin (100 mg/l), tetracyclin (50 mg/l) and ciprofloxacin (1.6 mg/l). The final solutions were titrated to pH 7.4 using NaOH. The voltage clamp experiments [57-59] were performed at room temperature 6 days after injection. Two-electrode voltage-clamp recordings were performed at a holding potential of -70 mV. The data were filtered at 10 Hz and recorded with a Digidata A/D-D/A converter (1322A Axon Instruments) and Clampex 9.2 software for data acquisition and analysis (Axon Instruments) [60-62]. The control superfusate contained 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂ and 5 mM HEPES, pH 7.4. Glutamate was added to the solutions at the indicated concentrations. The flow rate of the superfusion was approx. 20 ml/min, and a complete exchange of the bath solution was reached within about 10 s [63-65].

Detection of EAAT2 cell surface expression by chemiluminescence

For detection of EAAT2 cell surface expression, defolliculated oocytes were incubated during 1 hour at room temperature with rabbit anti- EAAT2 antibody (diluted 1:500, Alamone labs, Israel), washed and subsequently incubated with secondary, HRP-conjugated, goat anti-rabbit IgG antibody (1:1000, Cell Signaling technology, USA). After washing the second anti-body, oocytes were placed in 96 well plates for chemiluminescence assay with the SuperSignal ELISA Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL, USA), following the manufacturer's recommendations. Chemiluminescence of each oocyte was determined in a luminometer (Walter Wallac 2 plate reader, Perkin Elmer, Juegesheim, Germany) [36].

Statistical analysis

Data are provided as means ± SEM, n represents the number of oocytes investigated. All experiments were repeated with at least 3 batches of oocytes; in all repetitions qualitatively similar data were obtained. As expression of EAAT and/or mTOR may vary from batch to batch of oocytes, comparisons were always made

within the same oocyte batch [66, 67]. Data were tested for significance using ANOVA or t-test, as appropriate. Results with $p < 0.05$ were considered statistically significant.

Results

The present study analyzed the effect of mammalian target of rapamycin (mTOR) on glutamate transport by the excitatory amino acid transporters EAAT1 and EAAT2. To this end, cRNA encoding EAAT1 or EAAT2 was injected into *Xenopus laevis* oocytes with or without additional injection of cRNA encoding mTOR. Glutamate induced current determined by dual electrode voltage clamp was taken as measure of transport.

As shown in Fig. 1, addition of glutamate to the superfusate did not elicit an appreciable current in water-injected oocytes. Accordingly, the oocytes did not express significant endogenous electrogenic glutamate transport. In EAAT1 expressing oocytes, however, the addition of glutamate to the superfusate was followed by the appearance of a large inward current. The additional co-expression of wild-type mTOR was followed by a significant increase of glutamate induced current in EAAT1 expressing *Xenopus laevis* oocytes.

In order to test whether mTOR co-expression modifies the maximal glutamate induced current or the affinity of the carrier, the current induced by glutamate concentrations ranging from 1 μM to 5 mM was determined in *Xenopus laevis* oocytes expressing EAAT1 without or with additional expression of mTOR. As illustrated in Fig. 2, the glutamate induced current was a function of the extracellular glutamate concentration. Kinetic analysis revealed that the maximal glutamate induced current was significantly ($p < 0.001$) lower in *Xenopus laevis* oocytes expressing EAAT1 alone (71.92 ± 6.79 nA, $n = 7$) than in *Xenopus laevis* oocytes expressing EAAT1 together with mTOR (118.6 ± 6.0 nA, $n = 7$). The concentration required for half-maximal glutamate induced current was not significantly different between *Xenopus* oocytes expressing EAAT1 alone (379.51 ± 59.97 μM , $n = 7$) and *Xenopus* oocytes expressing EAAT1 together with wild-type mTOR (536.54 ± 112.07 μM , $n = 7$).

Similar observations were made with EAAT2. In EAAT2 expressing oocytes, the addition of glutamate to the superfusate was again followed by appearance of a large inward current (Fig. 3). The additional co-expression of wild-type mTOR was followed by a significant increase of glutamate induced current in EAAT2 expressing *Xenopus laevis* oocytes (Fig. 3).

Again, a kinetic analysis was made to test whether mTOR co-expression modifies the maximal glutamate induced current or the affinity of the carrier. To this end, the current

Fig. 1. Effect of mTOR on electrogenic glutamate transport in EAAT1 expressing *Xenopus laevis* oocytes. A: Representative original tracings showing glutamate (1 mM) - induced current (I_{EAAT}) in *Xenopus laevis* oocytes injected with water (a) or expressing EAAT1 (SLC1A3) without (b) or with (c) additional co-expression of mTOR. B: Arithmetic means $\pm$ SEM ($n = 14-16$) of I_{EAAT}

in *Xenopus laevis* oocytes injected with water (striated bar), or expressing EAAT3 without (white bar) or with mTOR (black bar). *** ($p < 0.001$) indicates statistically significant difference from the absence of mTOR.

Fig. 2. Electrogenic glutamate transport in EAAT1-expressing *Xenopus laevis* oocytes as a function of glutamate concentration without and with presence of mTOR. Arithmetic means $\pm$ SEM ($n = 7$) of I_{EAAT} as a function of glutamate concentration in *Xenopus laevis* oocytes expressing EAAT1 without (black squares), or with (black circles) additional co-expression of mTOR.

Fig. 3. Effect of mTOR on electrogenic glutamate transport in EAAT2 expressing *Xenopus laevis* oocytes. Arithmetic means $\pm$ SEM ($n = 11-15$) of I_{EAAT} in *Xenopus laevis* oocytes injected with water (striated bar), or expressing EAAT2 without (white bar) or with mTOR (black bar). *** ($p < 0.001$) indicates statistically significant difference from the absence of mTOR.

Fig. 4. Electrogenic glutamate transport in EAAT2-expressing *Xenopus laevis* oocytes as a function of glutamate concentration without and with presence of mTOR. Arithmetic means $\pm$ SEM ($n = 6-7$) of I_{EAAT} as a function of glutamate concentration in *Xenopus laevis* oocytes expressing EAAT2 without (black squares), or with (black circles) additional co-expression of wild-type mTOR.

induced by glutamate concentrations ranging from 1 μ M to 5 mM was determined in *Xenopus laevis* oocytes expressing EAAT2 without or with additional expression of mTOR. As illustrated in Fig. 4, the glutamate induced current was a function of the extracellular

Fig. 5. Effect of mTOR on EAAT2 abundance within the plasma membrane of oocytes. EAAT2 cell surface expression was assessed by chemiluminescence in *Xenopus* oocytes injected with water (striated bar), or with EAAT2 without (white bar) or with additional co-expression of mTOR (black bar). *** indicates statistically significant difference to oocytes expressing EAAT2 alone ($p < 0.001$) ($n = 51-58$). Data were normalized using the chemiluminescence signal of EAAT2 alone, as reference.

glutamate concentration. The calculated maximal glutamate induced current was significantly ($p < 0.001$) lower in *Xenopus laevis* oocytes expressing EAAT2 alone (62.79 ± 6.62 nA, $n = 6$) than in *Xenopus laevis* oocytes expressing EAAT2 together with wild-type mTOR (96.50 ± 7.98 nA, $n = 7$). The concentration required for half-maximal glutamate induced current was not significantly different between *Xenopus* oocytes expressing EAAT2 alone (512.75 ± 32.56 μ M, $n = 6$) and *Xenopus* oocytes expressing EAAT2 together with mTOR (489.70 ± 102.71 μ M, $n = 7$).

At least in theory, the increase of I_{glu} in EAAT2 expressing oocytes following coexpressing of mTOR could have resulted from an increase of carrier protein abundance in the cell membrane. Thus, chemiluminescence analysis was employed to test for altered carrier protein abundance within the cell surface. As illustrated in Fig. 5, the coexpression of mTOR indeed significantly increased the EAAT2 protein abundance in the cell membrane. The enhanced EAAT2 protein abundance in the cell membrane of EAAT2 and mTOR coexpressing oocytes could have resulted from either, accelerated insertion of new carriers into the cell membrane, or delayed clearance of carriers from the cell membrane.

Discussion

The present observations disclose a novel function of the mammalian target of rapamycin (mTOR), i.e. the up-regulation of the excitatory amino acid transporters EAAT1 and EAAT2. Coexpression of mTOR increased electrogenic glutamate transport in EAAT1 and EAAT2 expressing *Xenopus* oocytes. Coexpression of mTOR increased the maximal glutamate induced current in EAAT1 or EAAT2 expressing oocytes, without significantly modifying affinity of the carriers. According to chemiluminescence, mTOR increases the abundance of carrier protein in the plasma membrane.

By up-regulating the excitatory amino acid transporters, mTOR could accelerate the clearance of the excitatory amino acids glutamate and aspartate from the synaptic cleft and thus accelerate termination of excitation. Additional experimental effort is, however, required in order to define the functional significance of mTOR sensitive excitatory amino acid transport for the complex regulation of neuroexcitation.

In addition to neuronal, glial and sensory cells, EAAT1 is expressed in bone cells [68-71], and at least in theory, mTOR sensitive glutamate and/or aspartate transport may modify the function of those cells.

In conclusion, mTOR is a powerful stimulator of the excitatory amino acid transporters EAAT1 and EAAT2, an effect due to increase of carrier protein abundance in the cell membrane. The kinase may thus contribute to the fine tuning of neuronal excitation.

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Disclosure Statement

None.

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