Regulation of the Glutamate Transporter EAAT2 by PIKfyve

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Key Words

PIP5K3 • Astrocytes • Neuroexcitability • Glucocorticoids • Neurotransmission • Excitatory amino acid transporter 2

Abstract

The Na⁺/glutamate cotransporter EAAT2 is expressed in astrocytes and clears glutamate from the synaptic cleft. EAAT2 dependent current is stimulated by the serum and glucocorticoid inducible kinase SGK1. Phosphorylation targets of SGK1 include the human phosphatidylinositol-3-phosphate-5-kinase PIKfyve (PIP5K3). Nothing is known, however, on the role of PIKfyve in the regulation of EAAT2. The present experiments thus explored, whether PIKfyve expression modifies EAAT2 dependent current and protein abundance in the cell membrane. In *Xenopus* oocytes expressing EAAT2 but not in water injected oocytes application of glutamate (2 mM) induced an inward current (I_{glu}). Coexpression of either, SGK1 or PIKfyve, significantly enhanced I_{glu} in EAAT2 expressing oocytes. I_{glu} was significantly higher in *Xenopus* oocytes coexpressing EAAT2, SGK1 and PIKfyve than in *Xenopus* oocytes expressing EAAT2 and either, SGK1 or PIKfyve, alone. Additional

coexpression of the inactive mutant of the serum and glucocorticoid inducible kinase ^{K127N}SGK1 did not significantly alter I_{glu} in EAAT2 expressing oocytes and significantly decreased I_{glu} in oocytes coexpressing EAAT2 together with PIKfyve. The stimulating effect of PIKfyve on I_{glu} was abrogated by replacement of the serine in the SGK consensus sequence by alanine (^{S318A}PIKfyve). Furthermore, additional coexpression of ^{S318A}PIKfyve virtually abolished I_{glu} in *Xenopus* oocytes coexpressing SGK1 and EAAT2. Confocal microscopy reveals that PIKfyve enhances the EAAT2 protein abundance in the cell membrane. The observations disclose that PIKfyve indeed participates in the regulation of EAAT2.

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Introduction

The Na⁺/glutamate cotransporter EAAT2 is primarily expressed in astrocytes [1] and accomplishes glutamate reuptake from the synaptic cleft [2]. Upregulation of EAAT2 activity provides neuroprotection [3] and impaired expression or activity of EAAT2 leads to extracellular

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glutamate accumulation and neuroexcitotoxicity [4-7]. Accordingly, deranged EAAT2 function has been implicated in several neurological disorders including amyotrophic lateral sclerosis (ALS) [4, 8, 9], Alzheimer disease [10, 11], schizophrenia [12], HIV associated dementia [13], multiple sclerosis [14, 15], leukomalacia [16], epilepsy [17, 18], brain trauma [19], hypoxia and stroke [20-23]. Moreover, recent evidence points to an influence of gene variants in EAAT2 on reward dependence [24].

The role of EAAT2 in neuronal function and survival requires adequate regulation of the carrier by neuroexcitation. Regulators of EAAT2 include the serum and glucocorticoid inducible kinase SGK1 [25], a potent stimulator of several channels and transport proteins [26, 27]. The kinase may activate EAAT2 in part by preventing ubiquitination and degradation of the transporter by the ubiquitin ligase Nedd4-2 [25]. However, the fact that SGK1 modulates EAAT2 dependent current when coexpressed with a catalytically inactive Nedd4-2 mutant suggests that SGK1 might be effective through additional pathways. SGK1 phosphorylates and thus stimulates the mammalian phosphatidylinositol-3-phosphate-5-kinase PIKfyve [28], a kinase generating phosphatidylinositol 3,5-bisphosphate (PI(3,5)P₂) [29-32].

The present study explored the possibility, that PIKfyve participates in the regulation of EAAT2. To this end, EAAT2 was expressed in *Xenopus laevis* oocytes with or without PIKfyve. The results indeed reveal a stimulating effect of PIKfyve overexpression on carrier protein abundance and EAAT mediated current. Thus, PIKfyve presumably participates in the regulation of EAAT2.

Materials and Methods

Constructs

Constructs encoding wild type human EAAT2 [33], wild type PIKfyve [34], mutated ^{S318A}PIKfyve lacking the SGK phosphorylation consensus sequence [34], wild type human SGK1 [35], and inactive human ^{K127N}SGK1 [36] were utilized for generation of cRNA as described previously [37, 38]. EAAT2 cDNA was kindly provided by Susan Amara [39], ^{K127N}SGK1 cDNA was kindly provided by Sir Philip Cohen.

Electrophysiology

Xenopus laevis oocytes were prepared as previously described [38, 40, 41]. Five ng of wild type or mutant PIKfyve cRNA were injected on the first day and 1 ng EAAT2 cRNA on the second day after preparation of the oocytes. Where

indicated, 7.5 ng cRNA encoding SGK1 or ^{K127N}SGK1 were injected separately or together with PIKfyve. All experiments were performed at room temperature 4-5 days after the second injection. Two-electrode voltage-clamp recordings were performed at a holding potential of -60 mV. The data were filtered at 10 Hz, and recorded with a GeneClamp 500 amplifier, a DigiData 1322A A/D-D/A converter and the pClamp 9.0 software package for data acquisition and analysis (Axon Instruments, USA). The control solution (superfusate/ND96) 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 final solutions were titrated to pH 7.4 using NaOH. The flow rate of the superfusion was 20 ml/min and a complete exchange of the bath solution was reached within about 10 s.

Detection of cell surface expression by chemiluminescence

Defolliculated oocytes were first injected with either 7.5 ng SGK1 or 7.5 ng ^{K127N}SGK1 cRNA and one day later with 5 ng wild-type or 5 ng ^{S318A}PIKfyve and/or 1 ng EAAT2-HA which contains an HA epitope inserted extracellularly. Oocytes were incubated with 1 µg/mL primary rat monoclonal anti-HA antibody (clone 3 F10, Boehringer, Biberach, Germany) and 2 µg/mL secondary, peroxidase-conjugated affinity-purified F(ab')₂ goat anti-rat IgG antibody (Jackson ImmunoResearch, West Grove, PA, USA). Individual oocytes were placed in 20 µl of SuperSignal ELISA Femto Maximum Sensitivity Substrate (Pierce, Rockford, USA), and chemiluminescence was quantified in a luminometer (Walter Wallac 2 plate reader, Perkin Elmer, Juegesheim, Germany) by integrating the signal over a period of 1 s.

Immunofluorescence stainings

After 4% paraformaldehyde fixation for at least 12 h, oocytes were cryoprotected in 30% sucrose, frozen in Tissue Tek® O.C.T.™ Compound (Sakura Finetek) and placed on a cryostat. Sections were collected at a thickness of 8 µm on coated slides and stored at -20 °C. For immunostainings, sections were dehydrated at RT, fixated in acetone/methanol (1:1) for 15 minutes at RT, washed in PBS and pre-incubated for 1 h in 5% bovine serum albumin in PBS. The anti-HA EAAT2 antibody (clone 3 F10, Boehringer, Biberach, Germany) was diluted 1:100 and then applied in a moist chamber overnight at 4°C. Binding of anti-HA was visualized with an anti-rabbit conjugated Cy3 antibody (1:1000, donkey anti-rabbit, Millipore, United States). Slides were analyzed on a confocal laser-scanning microscope (Zeiss LSM 510 Exciter) using an appropriate filter set.

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. Data were tested for significance using ANOVA, and results with *P* < 0.05 were considered statistically significant.

Fig. 1. Coexpression of PIKfyve stimulates electrogenic glutamate transport in EAAT2 expressing *Xenopus laevis* oocytes. Arithmetic means $\pm$ SEM of glutamate (2 mM) induced normalized currents (I_{glu}) in oocytes injected with water (grey bar, n = 10), expressing EAAT2 without (white bar, n = 15) or with (black bar, n = 16) additional coexpression of PIKfyve, or expressing PIKfyve alone (hatched bar, n = 10). * indicates statistically significant difference to current in oocytes expressing EAAT2 alone.

Fig. 2. The effect of PIKfyve is augmented by SGK1. Arithmetic means $\pm$ SEM of glutamate (2 mM) induced normalized currents in *Xenopus laevis* oocytes expressing EAAT2 without (open bars) and with (closed bars) PIKfyve without (left bars, n = 16 and 16, resp.) or with (right bars, n = 16 and 16, resp.) additional expression of SGK1. * indicates statistically significant difference to oocytes expressing EAAT2 alone, # indicates significant difference to the respective values without additional expression of SGK1.

Results

PIKfyve stimulated electrogenic glutamate transport in EAAT2 expressing oocytes

Electrogenic glutamate transport was minimal in noninjected or water injected *Xenopus laevis* oocytes (Fig. 1). In oocytes expressing EAAT2, however, glutamate (2 mM) induced an inward current (I_{glu}) reflecting electrogenic entry of Na⁺ and glutamate. I_{glu} was significantly enhanced by additional expression of the phosphatidylinositol-3-phosphate-5-kinase PIKfyve (Fig. 1). Thus, PIKfyve stimulated EAAT2 dependent current.

The effect of PIKfyve was augmented by SGK1 in EAAT2 expressing oocytes

In oocytes expressing EAAT2 I_{glu} was stimulated by coexpression of the serum and glucocorticoid inducible kinase SGK1 (Fig. 2). The coexpression of SGK1 in addition to PIKfyve led to a significant further increase of I_{glu} in EAAT2 expressing oocytes. Moreover, I_{glu} was

significantly higher in oocytes coexpressing EAAT2, SGK1 and PIKfyve than in oocytes expressing EAAT2 and SGK1 alone.

Inactive ^{K127N}SGK1 did not stimulate electrogenic glutamate transport and reversed the stimulation by PIKfyve

In contrast to wild type SGK1, the inactive ^{K127N}SGK1 mutant did not significantly stimulate I_{glu} in oocytes expressing EAAT2. Injection of 7.5 ng cRNA encoding ^{K127N}SGK1 significantly blunted the stimulating effect of PIKfyve on I_{glu} . Coinjection of 25 ng of cRNA encoding ^{K127N}SGK1 completely reversed the stimulating effect of PIKfyve coexpression on I_{glu} in EAAT2 expressing oocytes, an effect again statistically significant (Fig. 3).

The effect of PIKfyve required an intact SGK1 phosphorylation consensus sequence

Replacement of the serine 318 with alanine in the only SGK1 phosphorylation consensus sequence within

Fig. 3. Coexpression of inactive ^{K127N}SGK1 does not stimulate EAAT2 and prevents stimulation by PIKfyve. Arithmetic means $\pm$ SEM of glutamate (2 mM) induced normalized currents in *Xenopus laevis* oocytes expressing EAAT2 without (open bars) and with (closed bars) PIKfyve. The currents were determined without (left bars, n = 21 and 24, resp.) or with (right bars, n = 11 and n = 20) additional expression of the inactive mutant ^{K127N}SGK1. * indicates statistically significant difference to oocytes expressing EAAT2 alone, # indicates significant difference to the respective value without additional expression of ^{K127N}SGK1.

Fig. 4. Replacement of serine318 by alanine abrogates the stimulating effect of PIKfyve on EAAT2. Arithmetic means $\pm$ SEM of glutamate (2 mM) induced normalized currents in *Xenopus laevis* oocytes expressing EAAT2 without (white bars) or with additional expression of the mutated ^{S318A}PIKfyve (black bars). The currents were determined without (left bars, n = 31 and 31, resp.) or with (right bars, n = 31 and 13, resp.) additional expression of wild type SGK1. * indicates statistically significant difference to oocytes expressing EAAT2 alone, # indicates statistically significant difference to the respective value in oocytes without expressing ^{S318A}PIKfyve.

the PIKfyve protein (^{S318A}PIKfyve) abrogated the stimulating effect on glutamate-induced current. I_{glu} was not significantly different between oocytes expressing EAAT2 alone and oocytes expressing EAAT2 together with ^{S318A}PIKfyve (Fig. 4). Coexpression of ^{S318A}PIKfyve significantly decreased the stimulating effect of SGK1 on I_{glu} (Fig. 4).

PIKfyve increases the EAAT2 protein abundance in the plasma membrane

PIKfyve and SGK1 could influence the EAAT2 mediated current by stimulating preformed protein or by enhancing the carrier protein abundance in the cell membrane. Thus, additional experiments were performed to quantify the membrane EAAT2 protein abundance. As a result, both confocal microscopy and chemiluminescence disclosed an increase of carrier protein abundance in the membrane of EAAT2 expressing

oocytes following coexpression of SGK1 or PIKfyve (Fig. 5). The combined coexpression of PIKfyve and SGK1 was followed by a further significant increase of EAAT2 protein abundance in the cell membrane.

Discussion

The present observations unravel a novel element in the regulation of EAAT2, the phosphatidylinositol-3-phosphate-5-kinase PIKfyve. Coexpression of PIKfyve is followed by a marked increase of glutamate induced currents in EAAT2 expressing oocytes. PIKfyve regulates endosomal transport [42–46] and is thus most likely effective through enhancing the carrier abundance at the cell surface. PIKfyve stimulates the formation of phosphatidylinositol 3,5-bisphosphate (PI(3,5)P₂), which is followed by PI(3,5)P₂-dependent docking of EAAT2

Fig. 5. PIKfyve enhances EAAT2 protein abundance in the plasma membrane. A. Confocal images of EAAT2 protein abundance in the plasma membrane of *Xenopus laevis* oocytes injected with water (left), EAAT2 alone (middle) and EAAT2 and PIKfyve (right). B. Chemiluminescence of oocytes injected with EAAT2 without (left bars) or with (right bars) SGK1 without (white bars) or with (black bars) coexpression of PIKfyve. * indicates statistically significant difference to oocytes expressing EAAT2 alone, # indicates significant difference to the chemiluminescence following expression of EAAT2 and PIKfyve alone.

containing membrane vesicles and subsequent insertion of the carrier into the cell membrane. Along those lines, PIKfyve enhances the EAAT2-protein abundance in the cell membrane of EAAT2 expressing *Xenopus laevis* oocytes, which fully explains the effect of the kinase on the glutamate induced currents. Nevertheless, the observation does not rule out involvement of further mechanisms in SGK1- or PIKfyve dependent regulation of EAAT2.

Earlier studies have shown that PIKfyve is a similarly potent stimulator of the glucose carrier GLUT4 [47, 48], the Na⁺/glucose cotransporter SGLT1 [49], the creatine transporter CreaT [50], the glutamate transporter EAAT3 [51] and the K⁺ channel KCNQ1/KCNE1 [28]. Thus, PIKfyve may be a regulator of a similarly wide range of carriers and channels as SGK1 [26]. However, unlike SGK1 [52], PIKfyve does not appreciably influence the intestinal phosphate transporter NaPiIb [51].

At least in theory, SGK1 and PIKfyve may be only effective when overexpressed. The relevance of SGK1 dependent transport regulation has been demonstrated by the respective phenotype of SGK1 knockout mice [26], indicating that at least SGK1 dependent transport regulation was correctly predicted from the voltage clamp

experiments in *Xenopus laevis* oocytes. A similar demonstration for the relevance of PIKfyve dependent transport regulation must await the availability of a PIKfyve knockout mouse.

As shown recently [28], SGK1 phosphorylates PIKfyve, an effect requiring an intact SGK1 consensus site. Replacement of the serine at the SGK1 consensus site with alanine yields the PIKfyve mutant ^{S318A}PIKfyve, which is expected to be resistant to regulation by SGK1. As shown here, ^{S318A}PIKfyve does not stimulate EAAT2. Accordingly, phosphorylation at the SGK1 consensus sequence appears to be required for the activation of the kinase. Coexpression of the inactive SGK1 mutant ^{K127N}SGK1, which lacks a functional kinase domain [36], does not stimulate EAAT2-dependent current, indicating that SGK1 is effective through phosphorylation. The effect of wild type PIKfyve does, however, not require the coexpression of SGK1. Thus, phosphorylation of PIKfyve by endogenous SGK1 or another kinase may be sufficient to activate PIKfyve in oocytes. Besides SGK1 protein kinase A has been shown to stimulate EAAT2 [53]. Coexpression of ^{K127N}SGK1 in sufficient quantity, however, significantly blunts or even fully reverses the stimulating effect of wild type PIKfyve.

Apparently, ^{K127N}SGK1 displaces the stimulating endogenous kinase from its binding site at PIKfyve and thus acts as a transdominant inhibitory kinase.

The effect of wild type SGK1 is similarly not dependent on the coexpression of wild type PIKfyve, indicating that again endogenous proteins could mediate the effect of SGK1. However, coexpression of ^{S318A}PIKfyve significantly blunts the effect of SGK1, i.e. again, the SGK1 insensitive ^{S318A}PIKfyve may compete with endogenous PIKfyve for SGK1 and thus exert a dominant negative effect. On the other hand, SGK1 may be effective through further cellular mechanisms. As shown earlier [25], SGK1 phosphorylates Nedd4-2, an ubiquitin ligase downregulating EAAT2. Thus, SGK1 may influence EAAT2 dependent current through more than one mechanism. The strong effect of ^{S318A}PIKfyve, however, strongly suggests that SGK1-dependent regulation of EAAT2 mainly involves this kinase. Notably, neither ^{K127N}SGK1, nor ^{S318A}PIKfyve decrease the

EAAT2-mediated current to values significantly below those in oocytes expressing EAAT2 alone. Thus, EAAT2 induced current appears to be partially SGK1- and PIKfyve- independent.

In conclusion, the Na⁺ coupled glutamate carrier EAAT2 is stimulated by PIKfyve, an effect requiring an intact SGK1 phosphorylation site. The present observations suggest that PIKfyve participates in the regulation of neuroexcitability.

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