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Regulation of the Glutamate Transporter EAAT4 by PIKfyve

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Key Words
PIP5K3 • EAAT4 • Purkinje cells • Neuroexcitability • Glucocorticoids • Neurotransmission

Abstract
The excitatory amino-acid transporter EAAT4 (SLC1A6), a Na^+,glutamate cotransporter expressed mainly in Purkinje cells, serves to clear glutamate from the synaptic cleft. EAAT4 activity is stimulated by the serum and glucocorticoid inducible kinase SGK1. SGK1-dependent regulation of the Na^+,glucose transporter SGLT1 (SLC5A1) and the creatine transporter CreaT (SLC6A8) has recently been shown to involve the mammalian phosphatidylinositol-3-phosphate-5-kinase PIKfyve (PIP5K3). The present experiments thus explored whether SGK1-dependent EAAT4-regulation similarly involves PIKfyve. In *Xenopus* oocytes expressing EAAT4, but not in water injected oocytes, glutamate induced a current which was significantly enhanced by coexpression of PIKfyve and SGK1. The glutamate induced current in *Xenopus* oocytes coexpressing EAAT4 and both, PIKfyve and SGK1, was significantly larger than the current in *Xenopus* oocytes expressing EAAT4 together with either kinase alone. Coexpression of the inactive SGK1 mutant ^{K127N}SGK1 did not significantly alter glutamate induced current in EAAT4-expressing *Xenopus* oocytes and abolished the stimulation of glutamate induced current by coexpression of PIKfyve. The stimulating effect of PIKfyve was abrogated by replacement of the serine with alanine in the SGK consensus sequence (^{S318A}PIKfyve). Furthermore, coexpression of ^{S318A}PIKfyve significantly blunted the stimulating effect of SGK1 on EAAT4 activity. The observations disclose that PIKfyve indeed participates in the regulation of EAAT4.

Introduction

The glutamate transporter EAAT4 (SLC1A6) is expressed in cerebellar Purkinje cells and is considered to clear glutamate from the synapses connecting the climbing fibers with the Purkinje cells [1, 2]. Recently, EAAT4 has been associated with schizophrenia [3, 4].

In view of its role in the regulation of extracellular glutamate concentrations and thus neuroexcitability, the carrier could be considered to be precisely regulated.

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As a matter of fact, several mechanisms have been identified, which regulate EAAT4 activity [5, 6], including the serum and glucocorticoid inducible kinase SGK1 [7]. The kinase regulates channels and carriers in part by direct phosphorylation and by interference with ubiquitination and subsequent degradation [8, 9].

Recently, a further mechanism of SGK1 dependent regulation of carriers has been identified. SGK1 phosphorylates the mammalian phosphatidylinositol-3-phosphate-5-kinase PIKfyve [10], a kinase generating phosphatidylinositol 3,5-bisphosphate ($PI(3,5)P_2$) [11-14]. PIKfyve has been shown to regulate endosomal transport [15-19]. As a result, PIKfyve has been shown to play a critical role in the regulation of the glucose carrier GLUT4 [20-22], the Na^+,glucose cotransporter SGLT1, the creatine transporter CreaT [24] and the K^+ channel KCNQ1/KCNE1 [10].

The present study explored, whether PIKfyve similarly participates in the regulation of EAAT4. To this end, EAAT4 has been expressed in *Xenopus* oocytes with or without PIKfyve and/or SGK1.

Materials and Methods

Constructs

For generation of cRNA, constructs were used encoding wild type rat EAAT4 (SLC1A6) [7], mutated ^{T40A}EAAT4 lacking the SGK1 phosphorylation consensus sequence, wild type murine PIKfyve [20, 25], mutated murine ^{S318A}PIKfyve lacking the SGK1 phosphorylation consensus sequence [10, 20], wild type human SGK1 [26], constitutively active human ^{S422D}SGK1 and inactive human ^{K127N}SGK1 [27]. The cRNA was generated as described previously [28, 29]. ^{K127N}SGK1 cDNA was kindly provided by Sir Philip Cohen, College of Life Sciences, Sir James Black Centre, University of Dundee.

Electrophysiology

Xenopus oocytes were prepared as previously described [29, 30]. 5 ng of wild type or mutant PIKfyve cRNA were injected on the first day and 25 ng EAAT4 or ^{T40A}EAAT4 cRNA on the second day after preparation of the *Xenopus* 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 recorded and filtered at 10 Hz, with a GeneClamp 500 amplifier and stored with 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 $CaCl_2$, 1 mM $MgCl_2$ 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 EAAT4 cell surface expression by chemiluminescence

Defolliculated oocytes were first injected with either 7.5 ng ^{S422D}SGK1 or 7.5 ng ^{K127N}SGK1 cRNA and one day later with 5 ng PIKfyve or 5 ng ^{S318A}PIKfyve and/or 25 ng EAAT4-HA, which contains an extracellular HA (hemagglutinin) epitope between aminoacids 374-375. 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 96 well plates with 20 µl of SuperSignal ELISA Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL, USA) and chemiluminescence of single oocytes was quantified in a luminometer (Walter Wallac 2 plate reader, Perkin Elmer, Juegesheim, Germany) by integrating the signal over a period of 1 s. Results display normalized relative light units.

Immunofluorescence in Xenopus oocytes

Oocytes for immunofluorescence were devitellinized and injected with 7.5 ng SGK1 and 5 ng PIKfyve (wt and S318A mutant), in different combinations. 5 days after injection, oocytes were fixed in 4% paraformaldehyde at room temperature for 2 h. After washing with phosphate buffered saline (PBS), oocytes were permeabilized and blocked at room temperature for 30 min by incubation in PBS containing 0,2% TritonX-100 and 10% normal goat serum. Then, the oocytes were incubated overnight at 4°C with different primary antibodies in order to detect $PI(3,5)P_2$, SGK1 and PIKfyve, respectively. In the case of $PI(3,5)P_2$ formation, a primary mouse monoclonal anti-$PI(3,5)P_2$ antibody was used (diluted 1:62, Echelon Biosciences Inc, Salt Lake City, USA) followed by 30 min incubation at 37°C with biotinylated goat anti-mouse IgG (diluted 1:5000, Jackson Immunoresearch, West Grove, PA, USA) in Tris buffered saline (TBS). After three times wash with TBS-Goat serum 1%, oocytes were incubated for 30 min at 37°C in Streptavidin-AlexaFluor 633 (diluted 1:2000, Invitrogen, Molecular Probes, Eugene, OR, USA) in TBS, washed with TBS and rinsed with distilled water. Other primary antibodies used were goat anti-SGK1 (diluted 1:50, Santa Cruz Biotechnologies, Heidelberg, Germany) and rabbit anti-PIKfyve (diluted 1:62, Abgent, San Diego, CA, USA). Incubation with the secondary antibodies was performed for 30 min at 37°C with the use of swine anti-goat IgG (H+L)-FITC (diluted 1:1000, Invitrogen, Molecular Probes, Eugene, OR, USA) and a goat anti-rabbit Cy3 AffiniPure (diluted 1:500, Jackson Immunoresearch, West Grove, PA, USA). Next, oocytes were analyzed by a fluorescence laser scanning microscope (LSM 510, Zeiss, Germany) with A-Plan 10x/0.25. Brightness and contrast settings were kept constant during imaging of all oocytes in each injection series. The quantification of the fluorescence intensity reflecting PI(3,5)P2 production was achieved by using ZEN2009 software (Zeiss, Germany).

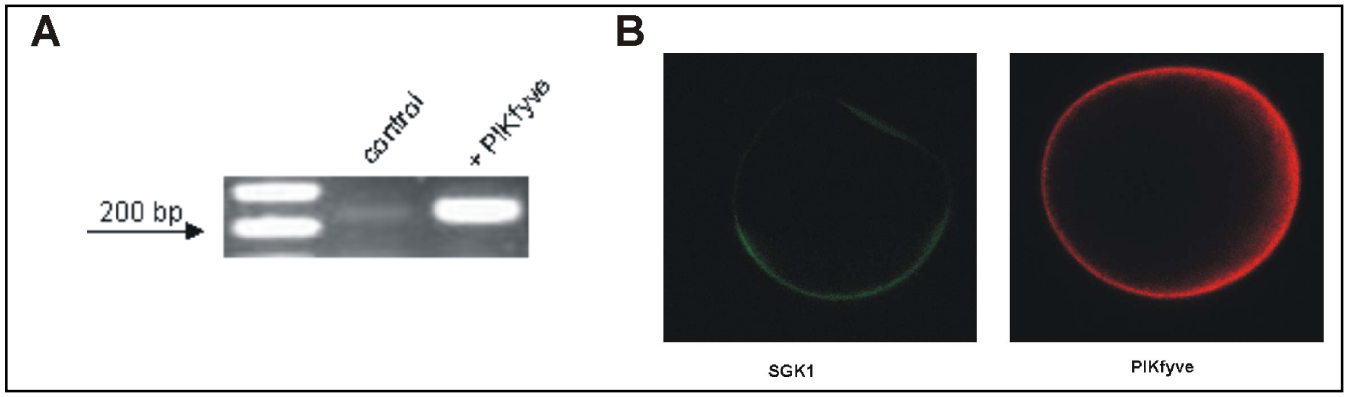

Fig. 1. PIKfyve is endogenously expressed in *Xenopus* oocytes and located in the plasma membrane. A. PIKfyve mRNA abundance without (left) and with (right) injection of exogenous PIKfyve. B. Confocal microsopy of the SGK1 (left panel) and PIKfyve (right panel) localization in *Xenopus* oocytes injected with wild type SGK1 (left panel) or PIKfyve (right panel).

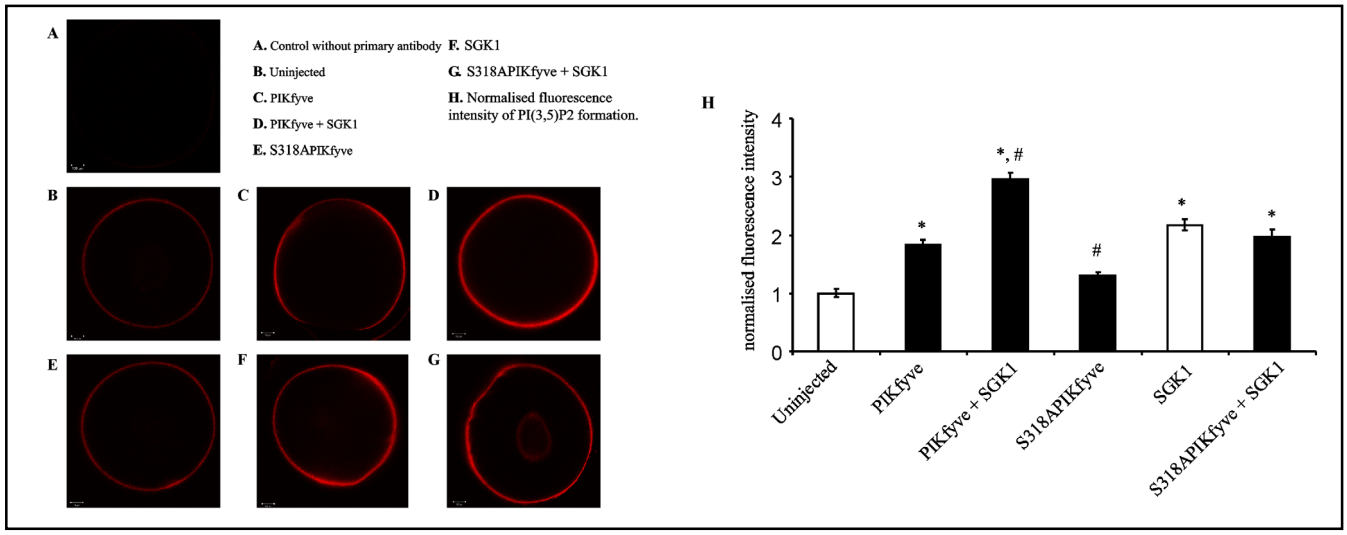

Fig. 2. $PI(3,5)P_2$ formation without and with expression of SGK1, of wild type or mutant PIKfyve. Confocal microsopy of the $PI(3,5)P_2$ abundance in *Xenopus* oocytes uninjected (B), injected with wild type PIKfyve without primary antibody (A), with wild type PIKfyve (C), with wild type PIKfyve + SGK1 (D), with inactive ^{S318A}PIKfyve (E); with wild type SGK1 (F) and with inactive ^{S318A}PIKfyve + SGK1 (G). PI(3,5)P2 formation was normalized to the mean fluorescence intensity values obtained in uninjected oocytes (H). * indicates statistically significant difference to uninjected oocytes, # indicates statistically significant difference to PIKfyve injected oocytes.

RT-PCR of endogenous PIKfyve

For the assessment of endogenously expressed PIKfyve, total RNA from control and injected oocytes was extracted and isolated using the Qiagen RNAeasy kit. Purified RNA was quantified by spectrophotometry. RT reactions were performed using 1µg of total RNA. The primers used to detect the endogenous PIKfyve were as follows: s: 5' GAT TTA GTG GTG TCC ATG GA 3', as: 5' CTA GCT GAG AAT GAT AAG CTA C 3'.

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.

Results

PIKfyve is expressed in Xenopus oocytes

PCR reactions, using as a template cDNA obtained from either uninjected or PIKfyve injected-oocytes, were performed in order to detect endogenous PIKfyve.

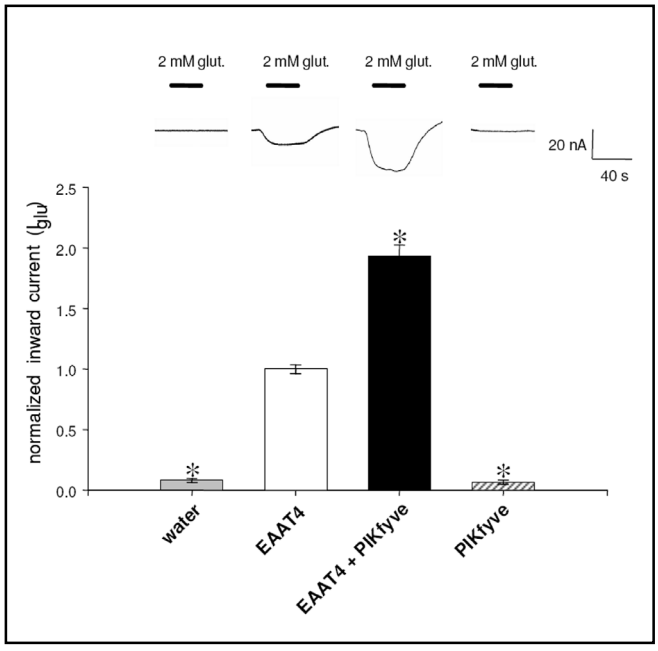

Fig. 3. Coexpression of PIKfyve stimulates electrogenic glutamate transport in EAAT4 expressing *Xenopus* oocytes. Arithmetic means ± SEM of glutamate (2 mM) induced normalized currents (I_{glu}) in *Xenopus* oocytes injected with water (grey bar, n = 10), expressing SLC1A6 (EAAT4) without (white bar, n = 10) or with (black bar, n = 10) additional coexpression of PIKfyve, or expressing PIKfyve alone (hatched bar, n = 10). *indicates statistically significant difference to current in *Xenopus* oocytes expressing EAAT4 alone.

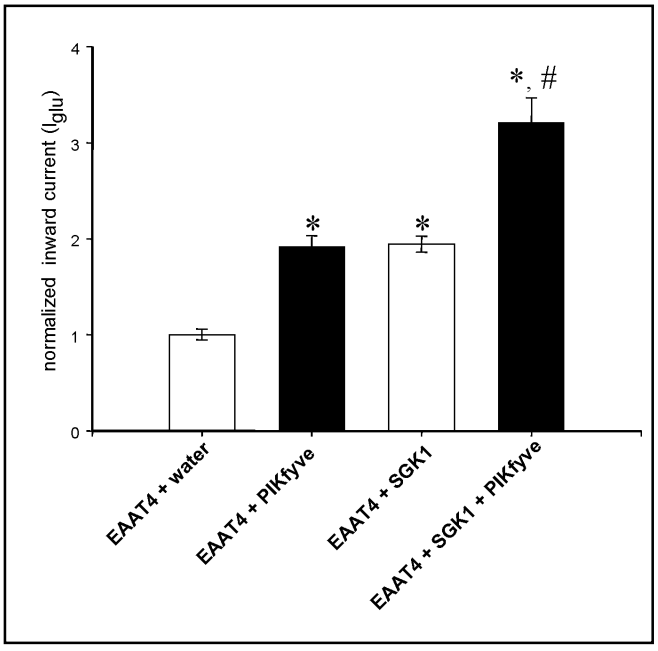

Fig. 4. The effect of PIKfyve is augmented by SGK1. Arithmetic means ± SEM of glutamate (2 mM) induced normalized currents (I_{glu}) in *Xenopus* oocytes expressing SLC1A6 (EAAT4) without (open bars) and with (closed bars) PIKfyve without (left bars, n = 36 and 31, resp.) or with (right bars, n = 37 and 27, resp.) additional expression of SGK1. *indicates statistically significant difference to *Xenopus* oocytes expressing EAAT4 alone, # indicates significant difference to the respective values without additional expression of SGK1.

As seen in Fig. 1A, endogenous PIKfyve is expressed in *Xenopus* oocytes. Confocal microscopy reveals cell membrane localization of both exogenous SGK1 and PIKfyve (Fig. 1B). As illustrated in Fig. 2, the expression of either, SGK1 or PIKfyve, modifies the abundance of $PI(3,5)P_2$ in the plasma membrane.

PIKfyve stimulated EAAT4 mediated electrogenic glutamate transport

Electrogenic glutamate transport was minimal in noninjected or water injected *Xenopus* oocytes (Fig. 3). In *Xenopus* oocytes expressing EAAT4 (SLC1A6), 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 mammalian phosphatidylinositol-3-phosphate-5-kinase PIKfyve (Fig. 3). Expression of PIKfyve alone did not lead to appreciable I_{glu}. Thus, PIKfyve stimulated EAAT4 activity.

The effect of PIKfyve was augmented by SGK1

I_{glu} in *Xenopus* oocytes expressing EAAT4 was further stimulated by coexpression of the serum and glucocorticoid inducible kinase SGK1 (Fig. 4). The coexpression of SGK1 in addition to EAAT4 and PIKfyve led to a significant further increase of I_{glu}. The I_{glu} was significantly larger in *Xenopus* oocytes expressing EAAT4, SGK1 and PIKfyve than in *Xenopus* oocytes expressing EAAT4 with either kinase alone.

Inactive ^{K127N}SGK1 did not stimulate electrogenic glutamate transport but blunted the stimulatory effect of PIKfyve

In contrast to wild type SGK1, the inactive ^{K127N}SGK1 mutant did not significantly stimulate I_{glu} in *Xenopus* oocytes expressing EAAT4 (Fig. 5). The I_{glu} in *Xenopus* oocytes coexpressing EAAT4 together with PIKfyve was significantly blunted by additional

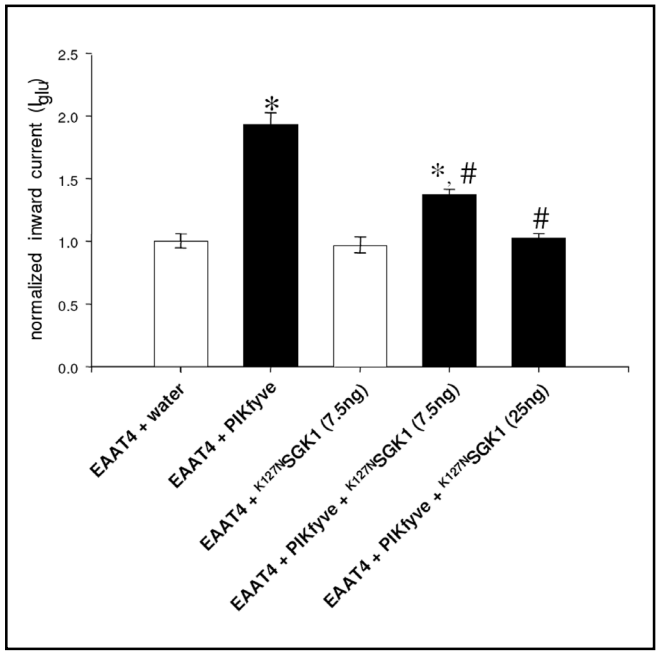

Fig. 5. Coexpression of inactive ^{K127N}SGK1 does not stimulate EAAT4 and blunts the stimulating effect of PIKfyve. Arithmetic means ± SEM of glutamate (2 mM) induced normalized currents (I_{glu}) in *Xenopus* oocytes expressing SLC1A6 (EAAT4) without (open bars) and with (closed bars) PIKfyve. The currents were determined without (left bars, n = 9 and 9, resp.) or with (right bars, n = 10, n= 15 and n=20) additional expression of the inactive mutant ^{K127N}SGK1. *indicates statistically significant difference to *Xenopus* oocytes expressing EAAT4 alone, # indicates significant difference to the respective value without additional expression of ^{K127N}SGK1.

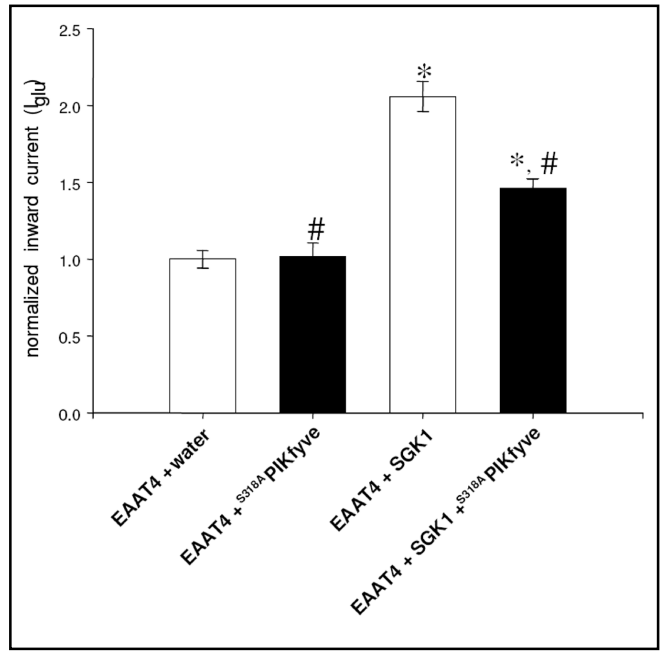

Fig. 6. Replacement of serine318 by alanine abrogates the stimulating effect of PIKfyve on EAAT4. Arithmetic means ± SEM of glutamate (2 mM) induced normalized currents (I_{glu}) in *Xenopus* oocytes expressing SLC1A6 (EAAT4) without (white bars) or with (black bars) additional expression of mutated ^{S318A}PIKfyve. The currents were determined without (left bars, n = 38 and 36, resp.) or with (right bars, n = 39 and 15, resp.) additional expression of wild type SGK1. *indicates statistically significant difference to *Xenopus* oocytes expressing EAAT4 alone, # indicates statistically significant difference to the respective value in *Xenopus* oocytes without expressing ^{S318A}PIKfyve.

coexpression of ^{K127N}SGK1. The stimulatory effect of PIKfyve was partially reversed by additional coexpression of 7.5ng ^{K127N}SGK1 and further blunted by coexpression of 25ng ^{K127N}SGK1 (Fig. 5).

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 the PIKfyve protein (^{S318A}PIKfyve) abrogated the stimulating effect on electrogenic glutamate transport (Fig. 6). Accordingly, I_{glu} was not significantly different between *Xenopus* oocytes expressing EAAT4 alone and *Xenopus* oocytes expressing EAAT4 together with ^{S318A}PIKfyve (Fig. 6). In *Xenopus* oocytes coexpressing EAAT4 together with SGK1, the additional coexpression of ^{S318A}PIKfyve was followed by a significant decrease of I_{glu}. In other words ^{S318A}PIKfyve partially reversed the stimulating effect of SGK1 on I_{glu} (Fig.6).

The stimulating effect of ^{S422D}SGK1 and/or PIKfyve required an intact SGK1 consensus site in EAAT4

Substitution of the threonine 40 with alanine in the SGK1 consensus site of EAAT4 abrogated the stimulating effect of SGK1 and PIKfyve on EAAT4. In *Xenopus* oocytes expressing ^{T40A}EAAT4 I_{glu} was not significantly modified by coexpression of either, SGK1 and PIKfyve (Fig. 7).

Coexpression of SGK1 or PIKfyve increased the plasma membrane abundance of EAAT4

Coexpression of EAAT4 with PIKfyve, with ^{S422D}SGK1 or with both kinases increased its membrane

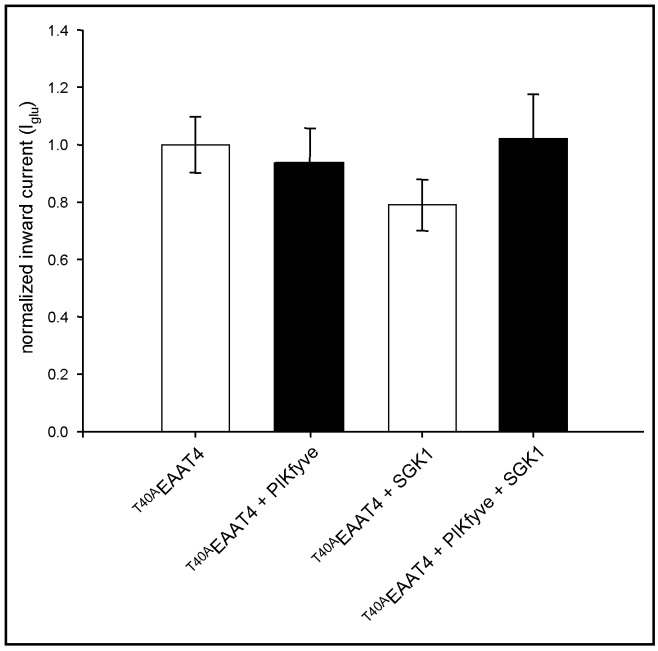

Fig. 7. Destruction of the SGK1 consensus site in the EAAT4 abolishes the stimulating effect of SGK1 and/or PIKfyve. Arithmetic means ± SEM of glutamate (2 mM) induced normalized currents (I_{glu}) in *Xenopus* oocytes expressing ^{T40A}SLC1A6 (^{T40A}EAAT4) without (open bars) and with (closed bars) PIKfyve without (left bars, n = 10 and 10, resp.) or with (right bars, n = 10 and 10, resp.) additional expression of SGK1. No statistically significant difference was obtained between the currents in the presence or absence of the kinases.

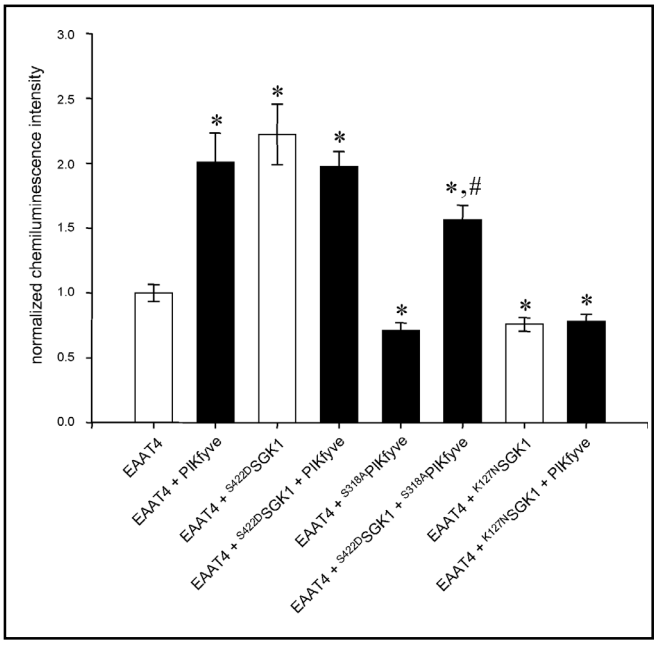

Fig. 8. Coexpression of PIKfyve and ^{S422D}SGK1 enhances the EAAT4 abundance within the plasma membrane of oocytes. Surface EAAT4 expression was assessed by chemiluminescence in oocytes expressing EAAT4 alone or together with PIKfyve (wt and/or ^{S318A} mutant) and/or SGK1 (wt or ^{S422D}SGK1 or ^{K127N}SGK1 mutants). Cell surface expression was normalized to the mean relative light units value obtained in oocytes expressing EAAT4 alone. *indicates statistically significant difference to *Xenopus* oocytes expressing EAAT4 alone, # indicates statistically significant difference to the group of *Xenopus* oocytes expressing EAAT4, ^{S422D}SGK1 and wild-type PIKfyve.

plasma abundance (Fig. 8), an effect abrogated by coexpression of EAAT4 with ^{S318A}PIKfyve or ^{K127N}SGK1.

Discussion

The present paper confirms the previously shown stimulating effect of the serum and glucocorticoid inducible kinase SGK1 on EAAT4 (SLC1A6) [7, 31]. More importantly, the present observations disclose a novel element in the regulation of EAAT4, i.e. the mammalian phosphatidylinositol-3-phosphate-5-kinase PIKfyve. The kinase is a potent stimulator of the Na^+,glutamate cotransporter EAAT4. In contrast to wild type PIKfyve, the SGK1 resistant PIKfyve mutant ^{S318A}PIKfyve does not stimulate EAAT4. Thus, SGK1-dependent regulation of EAAT4 may involve phosphorylation of PIKfyve by SGK1, formation of phosphatidylinositol 3,5-bisphosphate ($PI(3,5)P_2$) by PIKfyve and $PI(3,5)P_2$-dependent docking of EAAT4 containing membrane vesicles to the plasma membrane with subsequent insertion of the carrier into the cell membrane. Accordingly, the effect of PIKfyve is virtually abolished by coexpression of inactive ^{K127N}SGK1 and the effect of SGK1 is significantly blunted by coexpression of ^{S318A}PIKfyve. Those two observations point to interaction of the two kinases even though they do not rule out PIKfyve independent regulation of EAAT4 by SGK1 or other mechanisms of interaction between those two kinases.

The glutamate induced current is in EAAT4 + PIKfyve + ^{K127N}SGK1 injected oocytes smaller than in EAAT4 + PIKfyve injected oocytes. Along those lines, the current is smaller in EAAT4 + SGK1 + ^{S318A}PIKfyve injected oocytes than in EAAT4 + SGK1 injected oocytes. Those observations could be explained by the displacement of endogenous SGK by ^{K127N}SGK1 and of endogenous PIKfyve by ^{S318A}PIKfyve

from their respective targets. Thus, the inactive kinase mutants ^{K127N}SGK1 and ^{318A}PIKfyve may disrupt the function of the endogenous kinases and thus exert a transdominant inhibitory action.

EAAT4 has previously been shown to serve two functions, i.e. Na^+-coupled glutamate uptake and anion channel activity [32, 33]. The present study did not attempt to elucidate the anion channel acticity. The enhanced insertion of the EAAT4 protein into the cell membrane is expected, however, to increase both functions.

The regulation of the glutamate transporter by SGK1 and PIKfyve may be particularly relevant during cell shrinkage. SGK1 expression in Purkinje cells is weak under euvolemic conditions but heavily upregulated following dehydration [34]. SGK1 expression is upregulated by cell shrinkage [26] and excessive extracellular glucose concentrations [35, 36]. On the other hand, PIKfyve has been implicated in the generation of phosphatidylinositol 3,5-bisphosphate ($PI(3,5)P_2$) during cell shrinkage [14, 37]. SGK1 is regulated by the mammalian target of rapamycin mTOR [38, 39], which is again modified by cell volume [40]. Thus, at least in theory, SGK1 and PIKfyve could modify neuroexcitation during dehydration. It should be pointed out that SGK1 may modify neuro excitation in addition through regulation of further transporters for glutamate [41, 42], of transporters for further substrates [30, 43] and of channels [43-45].

In conclusion, the Na^+ coupled glutamate carrier EAAT4 is stimulated by PIKfyve, which thus mimics the stimulating effect of the serum and glucocorticoid inducible kinase SGK1. The present observations thus disclose a novel element in the regulation of synaptic glutamate concentration.

Acknowledgements

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