Original Paper

Up-Regulation of Amino Acid Transporter SLC6A19 Activity and Surface Protein Abundance by PKB/Akt and PIKfyve

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Key Words

Amino acid uptake • PKB/Akt • B⁰AT1 • PIKfyve

Abstract

Background: The amino acid transporter B⁰AT1 (SLC6A19) accomplishes concentrative cellular uptake of neutral amino acids. SLC6A19 is stimulated by serum- & glucocorticoid-inducible kinase (SGK) isoforms. SGKs are related to PKB/Akt isoforms, which also stimulate several amino acid transporters. PKB/Akt modulates glucose transport in part by phosphorylating and thus activating phosphatidylinositol-3-phosphate-5-kinase (PIKfyve), which fosters carrier protein insertion into the cell membrane. The present study explored whether PKB/Akt and/or PIKfyve stimulate SLC6A19. **Methods:** SLC6A19 was expressed in *Xenopus* oocytes with or without wild-type PKB/Akt or inactive T^{308A/S473A}PKB/Akt without or with additional expression of wild-type PIKfyve or PKB/Akt-resistant S^{318A}PIKfyve. Electrogenic amino acid transport was determined by dual electrode voltage clamping. **Results:** In SLC6A19-expressing oocytes but not in water-injected oocytes, the addition of the neutral amino acid L-leucine (2 mM) to the bath generated a current (I_{le}), which was significantly increased following coexpression of PKB/Akt, but not by coexpression of T^{308A/S473A}PKB/Akt. The effect of PKB/Akt was augmented by additional coexpression of PIKfyve but not of S^{318A}PIKfyve. Coexpression of PKB/Akt enhanced the maximal transport rate without significantly modifying the affinity of the carrier. The decline of I_{le} following inhibition of carrier insertion by brefeldin A (5 μM) was similar in the absence and presence of PKB/Akt indicating that PKB/Akt stimulated carrier insertion into rather than inhibiting carrier retrieval from the cell membrane. **Conclusion:** PKB/Akt up-regulates SLC6A19 activity, which may foster amino acid uptake into PKB/Akt-expressing epithelial and tumor cells.

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Introduction

The concentrative cellular uptake of several neutral amino acids including L-leucine into epithelial cells of the small intestine and of the proximal tubule of the kidney is accomplished by the Na⁺-coupled transporter B⁰AT1 (SLC6A19) [1-4]. Loss of function mutations of SLC6A19 lead to Hartnup disease, a classical autosomal recessive disorder with aminoaciduria resulting in several clinical symptoms such as photo-sensitive skin-rash, cerebellar ataxia, pellagra, dementia and late-onset seizures [3, 5-7]. SLC6A19 is under powerful regulation by the angiotensin-converting enzyme 2 (ACE2) and collectrin [3].

SLC6A19 has further been shown to be regulated by the serum- & glucocorticoid-inducible kinase isoforms SGK1-3 [8]. The SGKs share a variety of targets with PKB/Akt, which has previously been shown to stimulate the activity of several amino acid transporters [9-16]. PKB/Akt- and SGK-dependent regulation of transporters involves in part the phosphorylation and activation of the mammalian phosphatidylinositol-3-phosphate-5-kinase PIKfyve, a kinase generating phosphatidylinositol 3,5-bisphosphate (PI(3,5)P₂) [17-20]. PIKfyve regulates endosomal transport [21-25] and plays a critical role in the regulation of the glucose carrier GLUT4 [26-28], the Na⁺-glucose cotransporter SGLT1 [29], the creatine transporter CreaT [30], the Cl⁻ channels CFTR [31] and ClC2 [32], the Ca²⁺ channel TRPV6 [33], the K⁺ channel KCNQ1/KCNE1 [30] and the excitatory amino acid transporters [34, 35].

The present study explored, whether PKB/Akt regulates SLC6A19 and if so, whether PIKfyve is involved in this process. To this end, SLC6A19 activity was estimated from L-leucine-induced current utilizing dual electrode voltage clamp in *Xenopus* oocytes expressing SLC6A19 either alone or with wild-type PKB/Akt, inactive T^{308A}/S^{473A}PKB/Akt with or without additional expression of wild-type PIKfyve or PKB/Akt-resistant S^{318A}PIKfyve. As a result, coexpression of PKB/Akt but not of T^{308A}S^{473A}PKB/Akt enhanced electrogenic L-leucine transport in SLC6A19-expressing *Xenopus* oocytes, an effect augmented by additional expression of PIKfyve, but not of S^{318A}PIKfyve.

Materials and Methods

Constructs

For generation of cRNA the following cDNA constructs were used: Wild-type SLC6A19 [8], HA-tagged SLC6A19, wild-type PKB/Akt [31], inactive T^{308A}S^{473A}PKB/Akt [14], wild-type PIKfyve [26, 36], mutated S^{318A}PIKfyve lacking the SGK/PKB/Akt phosphorylation consensus sequence [26, 30] and wild-type Collectrin [8]. PKB/Akt cDNA was kindly provided by Sir Philip Cohen, College of Life Sciences, Sir James Black Centre, University of Dundee. HA-tagged SLC6A19 was generated by two-stage PCR site-directed mutagenesis using the primers:

SLC6A19-HA, S:

5' GTGAAGAGTGTTACGACGTACCAGATTACGCTGCCAAGAGCTC 3';

SLC6A19-HA, as:

5' GAGCTCTTGGC AGCGTAATCTGGTACGTCGTAACACTCTTCCAC 3'.

The mutant was sequenced to verify the presence of the desired mutation. The mutants were used for generation of cRNA as described previously [35].

Voltage clamp in *Xenopus* oocytes

For voltage clamp analysis, *Xenopus* oocytes were prepared as previously described [37]. On the day of preparation, *Xenopus laevis* oocytes were injected with water or cRNA encoding SLC6A19 (25 ng) with or without cRNA encoding collectrin (10 ng). One day later cRNA encoding wild-type PKB/Akt (10 ng), mutant T^{308A}/S^{473A}PKB/Akt (10 ng), wild-type PIKfyve (10 ng), mutant S^{318A}PIKfyve (10 ng) or dominant-negative PIKfyve^{K1831E} (10 ng) was injected. The oocytes were maintained at 17°C in ND96-A solution containing 88.5 mM NaCl, 2 mM KCl, 1.8 mM MgCl₂, 1 mM MgCl₂, 5 mM HEPES, 0.11 mM tetracycline, 4 μM ciprofloxacin, 0.2 mM gentamycin (Refobacin ©), 0.5 mM theophylline (Euphyllong ©) as well as 5 mM sodium pyruvate. The pH was adjusted to 7.4 by addition of NaOH. Where indicated, brefeldin A (5 μM, Sigma, Schnellendorf,

Germany) was added. The voltage-clamp experiments were performed at room temperature 3 days after the last injection [38]. Two-electrode voltage-clamp recordings were performed at a holding potential of -60 mV. Data were filtered at 10 Hz and recorded with Digidata 1322A converter and Clampex V.9 software for data acquisition and analysis (Axon Instruments, Union City, CA, USA). ND96 was used as the control superfusate (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1.0 mM MgCl₂, 5 mM HEPES, titrated to pH 7.4 by NaOH). L-leucine was added to the solutions at a concentration of 2 mM unless otherwise stated. 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 [39].

Detection of SLC6A19 cell surface expression by chemiluminescence

To determine SLC6A19 cell surface expression by chemiluminescence [34], oocytes were first incubated with 0.5 µg/mL primary rat anti-HA High Affinity antibody (Roche, Switzerland) and subsequently with a secondary, HRP-conjugated anti-rat IgG antibody (1:1000, Cell signaling Technology, MA, 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 sec. Results display normalized relative light units.

Statistical analysis

Data are provided as arithmetic means ± SEM; n represents the number of oocytes investigated. All oocyte experiments were repeated with at least three batches of oocytes and in all repetitions qualitatively similar data were obtained. All data were tested for significance by using ANOVA or t-test, as appropriate. Results with $p < 0.05$ were considered statistically significant.

Results

The present study explored the effect of PKB/Akt coexpression on SLC6A19 (B⁰AT)-dependent amino acid transport. To this end, cRNA encoding SLC6A19 was injected into *Xenopus* oocytes with or without additional injection of cRNA encoding PKB/Akt. Electrogenic amino acid transport was then determined with the dual electrode voltage clamp technique. Addition of L-leucine (2 mM) to the bath induced a small inward current in water-injected *Xenopus* oocytes, indicating that expression of endogenous electrogenic transport of L-leucine was low in *Xenopus* oocytes (Fig. 1). In *Xenopus* oocytes expressing SLC6A19, however, L-leucine (2 mM) induced an inward current (I_{le}) pointing to electrogenic entry of Na⁺ together with L-leucine (Fig. 1). As illustrated in Fig. 1, I_{le} was significantly enhanced by additional expression of PKB/Akt. In contrast to wild-type PKB/Akt, the inactive mutant ^{T308AS473A}PKB/Akt did not significantly modify the activity of SLC6A19 (Fig. 1).

As shown in Fig. 1C, the coexpression of collectrin resulted in a 17 ± 6 fold increase in I_{le} ($n = 9$). The additional coexpression of PKB/Akt in SLC6A19- and collectrin-expressing oocytes was followed by a further 45 ± 15 % ($n = 11$) increase in I_{le} . Thus, PKB/Akt increased SLC6A19-mediated transport both, in the presence and absence of collectrin.

According to kinetics analysis of L-leucine-induced currents in SLC6A19-expressing *Xenopus* oocytes (Fig. 2) the maximal current was 27.8 ± 0.2 nA and the L-leucine concentration required for halfmaximal current (K_M) approached 520 ± 22 µM, if SLC6A19 was expressed alone. The coexpression of SLC6A19 and PKB/Akt significantly increased the maximal current to 46.2 ± 0.4 nA but did not significantly alter K_M (576 ± 26 µM). Accordingly, coexpression of PKB/Akt enhanced SLC6A19 activity by increasing the maximal current without significantly influencing the affinity of the carrier.

As PKB/Akt is known to regulate GLUT4 via phosphorylation and thus activation of PIKfyve, additional experiments were performed exploring the impact of PIKfyve on SLC6A19 activity. As illustrated in Fig. 3, in *Xenopus* oocytes expressing SLC6A19 together with PKB/Akt, the additional coexpression of PIKfyve resulted in a further significant increase in I_{le} . In contrast, the additional expression of PKB/Akt-resistant ^{S318A}PIKfyve did not significantly modify I_{le} in SLC6A19- and PKB/Akt-coexpressing *Xenopus* oocytes. In the presence of

Fig. 1. Coexpression of PKB/Akt but not of T308A/S473A-PKB/Akt stimulated electrogenic L-leucine transport in SLC6A19-expressing *Xenopus* oocytes. A: Representative original tracings showing L-leucine (2 mM)-induced current (I_{le}) in *Xenopus* oocytes injected with water (a), expressing SLC6A19 without (b) or with (c) additional coexpression of wild-type PKB/Akt or of the inactive mutant T308A/S473A-PKB/Akt. B:

Arithmetic means $\pm$ SEM (n = 5-45) of the normalized L-leucine (2 mM)-induced current (I_{le}) in *Xenopus* oocytes injected with water (dotted bar), or expressing SLC6A19 without (white bar) or with additional coexpression of wild-type

PKB/Akt (black bar) or of the inactive mutant T308A/S473A-PKB/Akt (dark grey bar). *** (p<0.001) indicates statistically significant difference from expression of SLC6A19 alone. C: Arithmetic means $\pm$ SEM (n = 8-16) of the normalized L-leucine (2 mM)-induced current (I_{le}) in *Xenopus* oocytes injected with water (first dotted bar) or Collectrin (second dotted bar) or expressing SLC6A19 without (white bar) or with (black bar) PKB/Akt alone or coexpressed with Collectrin (dark grey bar). *** (p<0.001) indicates significant difference from the absence of PKB/Akt. # (p<0.05) indicates significant difference from the absence Collectrin.

Fig. 2. Coexpression of PKB/Akt enhanced the maximal L-leucine transport rate in SLC6A19-expressing *Xenopus* oocytes. Arithmetic means $\pm$ SEM (n = 14-19) of L-leucine-induced current (I_{le}) as a function of the extracellular L-leucine concentration in *Xenopus* oocytes expressing SLC6A19 without (white circles) and with PKB/Akt (black circles).

SLC6A19- and PKB/Akt the additional expression of S318A-PIKfyve tended to decrease I_{le} , an effect, however, not reaching statistical significance. Thus, it remains uncertain, whether or not S318A-PIKfyve exerts a dominant negative effect by displacing a putative *Xenopus* PIKfyve homologue, which could, in theory, mediate the effect of PKB in the absence of exogenous wild type PIKfyve.

The enhanced maximal SLC6A19 activity following PKB/Akt coexpression could result from increased carrier protein abundance in the plasma membrane. To explore this possibility, chemiluminescence experiments were performed. As illustrated in Fig. 4, the coexpression of PKB/Akt resulted in a significant increase in the SLC6A19 protein abundance within the oocyte plasma membrane.

Fig. 3. The effect of PKB/Akt was augmented by wild-type PIKfyve but not by PKB/Akt-resistant S^{318A} PIKfyve. A: Representative original tracings showing L-leucine (2 mM)-induced current (I_{le}) in *Xenopus* oocytes injected with water (a), expressing SLC6A19 alone (b), or coexpressing SLC6A19 with PKB/Akt (c-e) without (c) or with additional expression of wild-type PIKfyve (d) or of PKB/Akt-resistant S^{318A} PIKfyve (e). B: Arithmetic means $\pm$ SEM (n = 12-19) of the normalized L-leucine (2 mM)-induced current (I_{le}) in *Xenopus* oocytes injected with water (dotted bar), or expressing SLC6A19 without (white bar) or with PKB/Akt cRNA without (black bar) or with additional coexpression of wild-type PIKfyve (dark grey bar) or of PKB/Akt-resistant S^{318A} PIKfyve (light grey bar). ** (p<0.01) indicates significant difference

from the absence of PKB/Akt. # (p<0.05) indicates significant difference from the absence of wild-type PIKfyve. C: Arithmetic means $\pm$ SEM (n = 5-11) of the normalized L-leucine (2 mM)-induced current (I_{le}) in *Xenopus* oocytes injected with water (dotted bar), or expressing SLC6A19 without (white bar) or with PKB/Akt cRNA without (black bar) or with additional coexpression of wild-type PIKfyve (dark grey bar) or a dominant-negative PIKfyve K^{1831E} mutant (light grey bar). *, *** (p<0.05, p<0.001) indicates significant difference from the absence of PKB/Akt. # (p<0.05) indicates significant difference from the absence of wild-type PIKfyve.

Fig. 4. Coexpression of PKB/Akt enhanced surface SLC6A19 protein abundance in SLC6A19-expressing *Xenopus* oocytes. A: Arithmetic means $\pm$ SEM (n = 52-67) of the normalized chemiluminescence signal of HA-tagged SLC6A19 in *Xenopus* oocytes injected with water (dotted bar), or expressing SLC6A19 without (white bar) or with (black bar) additional coexpression of wild-type PKB/Akt. *** (p<0.001) indicates statistically significant difference from the absence of PKB/Akt.

In theory, PKB/Akt could stimulate SLC6A19 by promoting the insertion of carrier protein into the membrane or by delaying carrier retrieval from the membrane. To discriminate between these two possibilities, SLC6A19-expressing *Xenopus* oocytes were treated with 5 μ M brefeldin A, an inhibitor of carrier insertion into the cell membrane. As shown in Fig. 5, the L-leucine-induced current declined in the presence of brefeldin A at a similar rate in oocytes expressing SLC6A19 alone and in oocytes expressing SLC6A19

Fig. 5. Effect of brefeldin A on *Xenopus* oocytes expressing either SLC6A19 alone or with PKB/Akt. Arithmetic means $\pm$ SEM (n = 22–32) of the normalized L-leucine (2 mM)-induced current (I_{le}) in *Xenopus* oocytes injected with water (dotted bar) or expressing HA-tagged SLC6A19 without (white bars) or with (black bars) PKB/Akt and incubated in the presence (right bars) or absence (left bars) of 5 μ M

brefeldin A for 24 hours prior to the measurement. *** (p<0.001) indicates significant difference from the absence of PKB/Akt. ### (p<0.001) indicates significant difference from the absence of brefeldin A.

together with PKB/Akt. In addition, in the presence of brefeldin A, coexpression of PKB/Akt failed to enhance SLC6A19 activity (Fig. 5). Accordingly, retrieval of carrier protein from the cell membrane was not significantly different between *Xenopus* oocytes expressing SLC6A19 alone and *Xenopus* oocytes expressing SLC6A19 together with PKB/Akt. Therefore, PKB/Akt increased SLC6A19 activity by stimulating carrier protein insertion into rather than by delaying retrieval of carrier protein from the cell membrane.

Discussion

The present study reveals a novel function of PKB/Akt, i.e. the up-regulation of the neutral amino acid transporter SLC6A19. PKB/Akt, but not its inactive mutant ^{T308A/S473A}PKB/Akt enhanced the maximal transport rate of the carrier. The effect is augmented by additional coexpression of PIKfyve but not of PKB/Akt-resistant ^{S318A}PIKfyve, suggesting that PKB/Akt may at least partially be effective through activation of PIKfyve. In view of the decay of L-leucine-induced current following treatment of the *Xenopus* oocytes with brefeldin A, PKB/Akt appears to stimulate carrier insertion rather than delaying carrier retrieval from the cell membrane. In contrast, SGK1-3 promote SLC6A19 surface abundance by stabilizing the transporter at the plasma membrane [8].

SLC6A19 is dramatically up-regulated by ACE2 and collectrin [40–42], proteins associating with SLC6A19 [40, 42]. SGK1-3 activate SLC6A19 when coexpressed with ACE2, but do not when coexpressed with collectrin [8]. As shown here, PKB/Akt does stimulate transport in oocytes expressing both, SLC6A19 and collectrin. The effect of PKB/Akt is moderate, however, in comparison to the powerful effect of collectrin.

L-Leucine uptake via Na⁺-coupled transport results in entry of the respective amino acids together with Na⁺. The Na⁺ taken up is extruded by the Na⁺/K⁺ ATPase. Thus SLC6A19 activity results in cellular accumulation of amino acids along with K⁺ and osmotically obliged water and thus in cell swelling [43]. Accordingly, the upregulation of Na⁺-coupled amino acid transport by PKB/Akt may represent a challenge for cell volume regulation. PKB/Akt activation has indeed been shown to increase cell size [44], an effect, which may not only be based on the stimulation of amino acid uptake, but as well on activation of further carriers resulting in cellular ion accumulation, such as the Na⁺/H⁺ exchanger [45].

Cellular uptake of amino acids may foster survival of proliferating cells [46, 47]. Several amino acid transporters have been reported to be critically important for survival and proliferation of cancer cells [48]. Cellular amino acid uptake may stimulate tumor growth by regulating mammalian target of rapamycin (mTOR) through the nutrient pathway [49]. The expression of various amino acid transporters is elevated in a wide spectrum of primary human cancers and may play a critical role in the regulation of growth and survival of cancer cells [48, 50]. Thus, amino acid transporters are considered potential targets for cancer

therapy [50]. Along those lines, stimulation of amino acid transport may contribute to the well known tumor-promoting effect of PKB/Akt activity [51-59].

In conclusion, PKB/Akt stimulates the Na⁺-coupled amino acid transporter SLC6A19 (B⁰AT) by promoting carrier insertion into the plasma membrane, an effect possibly involving PIKfyve. Stimulation of SLC6A19 by PKB/AKT may contribute to the regulation of amino acid uptake in intestinal and renal epithelial cells as well as cancer cells.

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