PIKfyve in the SGK1 Mediated Regulation of the Creatine Transporter SLC6A8

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Key Words

PIP5K3 • Kinase • PI(3,5)P₂ • CreaT • Kidney • Heart • Brain • Skeletal muscle

Abstract

The Na⁺,Cl⁻creatine transporter CreaT (SLC6A8) mediates concentrative cellular uptake of creatine into a wide variety of cells. Previous observations disclosed that SLC6A8 transport activity is enhanced by mammalian target of rapamycin (mTOR) at least partially through the serum and glucocorticoid inducible kinase isoforms SGK1 and SGK3. As SLC6A8 does not contain a putative SGK consensus motif, the mechanism linking SGK1 with SLC6A8 activity remained elusive. A candidate kinase is the mammalian phosphatidylinositol-3-phosphate-5-kinase PIKfyve (PIP5K3), which has previously been shown to regulate the glucose transporter GLUT4. The present experiments explored the possibility that SLC6A8 is regulated by PIKfyve. In *Xenopus* oocytes expressing SLC6A8 but not in water injected oocytes creatine induced a current which was significantly enhanced by coexpression of PIKfyve. The effect of

PIKfyve on SLC6A8 was blunted by additional coexpression of the inactive mutant of the serum and glucocorticoid inducible kinase ^{K127N}SGK1. The stimulating effect of PIKfyve was abrogated by replacement of the serine in the SGK consensus sequence by alanine (^{S318A}PIKfyve). Moreover, coexpression of ^{S318A}PIKfyve blunted the effect of SGK1 on SLC6A8 activity. The observations suggest that SGK1 regulates the creatine transporter SLC6A8 at least partially through phosphorylation and activation of PIKfyve and subsequent formation of PI(3,5)P₂.

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Introduction

The creatine transporter CreaT (SLC6A8), a member of the superfamily of Na⁺,Cl⁻ coupled transporters of various neurotransmitters and of amino acids, accomplishes the cellular accumulation of creatine [1, 2]. SLC6A8 is expressed in a wide variety of tissues including skeletal muscle, kidney, small intestine, heart, brain and

retina [3]. Creatine reversibly binds phosphate and may thus replenish ATP at short term energy depletion [4]. Its accumulation supports the cellular ability to store metabolic energy. Genetic defects of the carrier lead to mental retardation with seizures [5, 6].

Recently, the serum and glucocorticoid inducible kinase isoforms SGK1 and SGK3 [7] and the mammalian target of rapamycin [8] have been identified as major stimulators of SLC6A8. SGK1 is transcriptionally regulated by a wide variety of hormones and stress conditions [9, 10]. As SLC6A8 lacks the putative SGK1/SGK3 phosphorylation consensus motifs (RXRXXS/T and RRXS/T), SGK1 stimulates the carrier presumably not by direct phosphorylation. Alternatively, SGK1 could stimulate SLC6A8 through phosphorylation and stimulation of a downstream signaling molecule. A candidate target is the mammalian phosphatidylinositol-3-phosphate-5-kinase PIKfyve, which has previously been shown to be phosphorylated and thus activated by PKB [11]. SGK isoforms and PKB share the same consensus sequence [12] and thus phosphorylate similar proteins, with the notable exception of NDRG1 (N-myc downstream-regulated gene 1) [13]. The present study thus explored whether PIKfyve could participate in the SGK1-dependent regulation of SLC6A8 activity.

Materials and Methods

Constructs

For generation of cRNA, constructs were used encoding wild type bovine CreaT (SLC6A8) [1], wild type PIKfyve [11, 14], mutated ^{S318A}PIKfyve lacking the SGK phosphorylation consensus sequence [11, 14], wild type human SGK1 [15, 16], and inactive human ^{K127N}SGK1 [17], as described previously. ^{K127N}SGK1 was kindly provided by Sir Philip Cohen.

Electrophysiology

Xenopus oocytes were prepared as previously described [18, 19]. 5 ng of PIKfyve cRNA were injected on the first day and 15 ng SLC6A8 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 5-6 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 Digidata A/D-D/A converter and Chart V.4.2 software for data acquisition and analysis (Axon Instruments). 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. Creatine was added to the solutions at the indicated concentrations. The final solutions were titrated to pH 7.4 using

Fig. 1. Coexpression of PIKfyve stimulates electrogenic creatine transport in SLC6A8 expressing *Xenopus* oocytes. Arithmetic means $\pm$ SEM of creatine (50 μ M) induced current (I_{CREA}) in *Xenopus* oocytes injected with water (light grey bar, $n = 11$), expressing SLC6A8 alone (open bar, $n = 27$), expressing PIKfyve alone (dark grey bar, $n = 9$), or expressing SLC6A8 together with PIKfyve (black bar, $n = 28$). * indicates statistically significant difference to current in *Xenopus* oocytes expressing SLC6A8 alone.

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.

Statistical analysis

Data are provided as means $\pm$ 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 increased creatine induced current in SLC6A8 expressing *Xenopus* oocytes

In *Xenopus* oocytes expressing bovine SLC6A8 but not in water injected oocytes creatine induced an inward current (I_{CREA}), which was significantly increased by additional coexpression of PIKfyve (Fig. 1). I_{CREA} tended to be higher in *Xenopus* oocytes expressing PIKfyve as compared to oocytes injected with water, a difference, however, not reaching statistical significance (Fig. 1). Accordingly, upregulation of endogeneous carriers by PIKfyve does not significantly contribute to the stimulation of I_{CREA} in SLC6A8 expressing oocytes.

Fig. 2. The effect of PIKfyve is mimicked and potentiated by SGK1. Arithmetic means $\pm$ SEM of I_{CREA} in *Xenopus* oocytes expressing SLC6A8 without (open bars) and with PIKfyve (closed bars). The currents were determined without (left bars, $n = 5$ and 5 , resp.) or with (right bars, $n = 5$ and 4 , resp.) additional expression of wild type SGK1. *indicates statistically significant difference between *Xenopus* oocytes expressing PIKfyve together with SLC6A8 and *Xenopus* oocytes expressing SLC6A8 alone, # indicates statistically significant difference between the respective values with and without additional expression of SGK1.

The effect of PIKfyve was mimicked by SGK1

Coexpression of the serum and glucocorticoid inducible kinase SGK1 with SLC6A8 was similarly capable to enhance the creatine induced currents (I_{CREA}) as coexpression of PIKfyve with SLC6A8. As illustrated in Fig. 2, coexpression of SGK1 significantly increased I_{CREA} . The coexpression of both, PIKfyve and SGK1 was followed by a further increase of I_{CREA} .

The effect of PIKfyve was reversed by inactive K^{127N} SGK1

Coexpression of the inactive mutant serum and glucocorticoid inducible kinase K^{127N} SGK1 did not stimulate the creatine induced currents (I_{CREA}) in SLC6A8 expressing *Xenopus* oocytes. As shown in Fig. 3, no significant difference was observed between I_{CREA} in *Xenopus* oocytes expressing SLC6A8 alone and *Xenopus* oocytes expressing both, SLC6A8 and K^{127N} SGK1. In oocytes expressing both, SLC6A8 and PIKfyve, the additional expression of K^{127N} SGK1 significantly decreased I_{CREA} .

Fig. 3. The effect of PIKfyve is reversed by inactive K^{127N} SGK1. Arithmetic means $\pm$ SEM of I_{CREA} in *Xenopus* oocytes expressing SLC6A8 without (open bars) and with PIKfyve (closed bars). The currents were determined without (left bars, $n = 7$ and 6 , resp.) or with (right bars, $n = 5$ and 6 , resp.) additional expression of inactive mutant K^{127N} SGK1. *indicates statistically significant difference between *Xenopus* oocytes expressing PIKfyve together with SLC6A8 and *Xenopus* oocytes expressing SLC6A8 alone, # indicates statistically significant difference between the respective values with and without additional expression of K^{127N} SGK1.

The effect of PIKfyve was abrogated by mutation at the SGK1 phosphorylation consensus sequence

The stimulating effect of PIKfyve was abrogated by replacement of the serine in the putative SGK consensus sequence by alanine (S^{318A} PIKfyve). Coexpression of S^{318A} PIKfyve did not significantly increase I_{CREA} in SLC6A8 expressing *Xenopus* oocytes. Moreover, S^{318A} PIKfyve blunted the stimulating effect of SGK1 on creatine transport. In oocytes coexpressing both, SLC6A8 and SGK1, the additional coexpression of S^{318A} PIKfyve significantly decreased I_{CREA} (Fig. 4).

Discussion

The present study demonstrates that PIKfyve stimulates the creatine transporter CreaT (SLC6A8), an effect mimicking the stimulating effect of the serum and glucocorticoid inducible kinase [7]. The effect of SGK1

Fig. 4. Replacement of serine318 by alanine in PIKfyve abrogates the stimulating effect of PIKfyve or SGK1 on SLC6A8. Arithmetic means $\pm$ SEM of I_{CREA} in *Xenopus* oocytes expressing SLC6A8 without (open bars) and with wild type PIKfyve (closed bars) or with $\text{S}^{318\text{A}}$ PIKfyve (grey bars). The currents were determined without (left bars, $n = 6, 4$ and 5 , resp.) or with (right bars, $n = 4, 5$ and 5 , resp.) additional expression of wild type SGK1. *indicates statistically significant difference to *Xenopus* oocytes expressing SLC6A8 alone, # indicates statistically significant difference to the respective values without additional expression of SGK1.

Fig. 5. Schematic diagram of regulation of endosomal trafficking of CreaT(SLC6A8). Schematic diagram showing a putative mechanism regulating endosomal trafficking of CreaT(SLC6A8) between the plasma membrane and recycling endosomes. Activated of mammalian target of rapamycin (mTOR), insulin or growth factors stimulate the serum and glucocorticoid inducible kinase SGK1. The kinase in turn phosphorylates and thus activates PIKfyve thus leading to enhanced formation of $\text{PI}(3,5)\text{P}_2$, which increases SLC6A8 trafficking to the plasma membrane. A similar mechanism was recently suggested for the ion channel complex KCNQ1/KCNE1 [14].

is at least partially reversed by the mutant $\text{S}^{318\text{A}}$ PIKfyve and the effect of PIKfyve is abrogated by the inactive $\text{K}^{127\text{N}}$ SGK1.

Both observations suggest that SGK1 and PIKfyve share the same signaling pathway. As coexpression of SGK1 enhances creatine induced currents (I_{CREAT}) in SLC6A8 expressing oocytes (Fig. 2, Fig. 4), the kinase may accomplish its stimulation in part by an additional PIKfyve-independent mechanism. At least in theory, this mechanism could involve direct phosphorylation at other, non-canonical, SGK1 consensus sequences. Alternatively, SGK1 regulates SLC6A8 through endogenously

expressed PIKfyve. The inhibitory effect of $\text{S}^{318\text{A}}$ PIKfyve is suggestive for a mechanism depending on endogenous PIKfyve, which is displaced by the inactive mutant $\text{S}^{318\text{A}}$ PIKfyve. Along those lines, currents in oocytes overexpressing SLC6A8 alone could result from regulation by endogenous PIKfyve and/or endogenous SGK1, or from SLC6A8 regulating mechanisms not requiring interaction of SGK1 and PIKfyve (Fig. 1-4).

We previously showed that SGK1 phosphorylates PIKfyve to produce $\text{PI}(3,5)\text{P}_2$. [14]. This phospholipid is a potent modulator of vesicle recycling which stimulates recycling vesicle exocytosis in oocytes and mammalian cells

[14]. These RAB11A positive vesicles also contain KCNQ1/KCNE1 channels and result in increased plasma membrane abundance of the channels [14]. It is well possible that a similar mechanism underlies SLC6A8 stimulation (Fig. 5).

The present observations may be relevant for the regulation of further transport systems. SGK1 enhances the activity and cell membrane abundance of a wide variety of ion channels and transporters [10] including the epithelial Na⁺ channel ENaC [20-22], the voltage gated K⁺ channels KCNE1/KCNQ1 [14, 23], KCNQ4 [18], and HERG [24], the Ca²⁺ channel TRPV5 [15], the glutamate receptors GluR1 and GluR6 [25, 26], the Na⁺ coupled glucose transporter SGLT1 [27], the amino acid transporters SN1 [28], EAAT1 [29], EAAT3 [30], EAAT4 [31] and EAAT5 [32], the creatine transporter CreaT [7], and the phosphate transporter NaPiIb [33]. Moreover, stimulation of SLC6A8 by SGK1 is involved in the regulation of the carrier by the mammalian target of rapamycin mTOR [8]. Thus, the present signaling cascade might mediate part of the mTOR-dependent transport regulation.

The signaling cascade may be particularly important for cell volume sensitive transport regulation. SGK1 expression is highly sensitive to cell volume and markedly upregulated by osmotic and isotonic cell shrinkage [16]. mTOR has in turn been shown to influence cell volume [34]. Moreover, SLC6A8 is closely related to the osmolyte transporters BGT and TAUT [35].

The observed signaling cascade may participate in

regulation of creatine transport in a variety of physiological and pathophysiological conditions. SGK1 expression is upregulated by a wide variety of conditions including increased cytosolic Ca²⁺ activity [36], ischemia [37] and several hormones such as glucocorticoids [9], mineralocorticoids [20, 21], or TGF- β [38]. SLC6A8 abundance is decreased in the failing heart [39] and defective SLC6A8 has been shown to cause mental retardation and epilepsy [5, 6].

In conclusion, the present and previous observations disclose a signaling cascade regulating cellular creatine uptake. Activation of mammalian target of rapamycin (mTOR) on the one hand, and insulin or growth factors such as IGF1 on the other, activate the serum and glucocorticoid inducible kinase SGK1. The kinase in turn phosphorylates and thus activates PIKfyve thus leading to enhanced formation of PI(3, 5)P₂, which enhances SLC6A8 trafficking to the plasma membrane (Fig. 5). Genomic upregulation of SGK1 by cell stress or hormones [10] amplifies the power of this signaling cascade and thus allows the cellular adaptation to physiological and pathophysiological challenges.

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