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Downregulation of the osmolyte transporters SMIT and BGT1 by AMP-activated protein kinase

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ABSTRACT

The myoinositol transporter SMIT (SLC5A3) and the betaine/γ-aminobutyric acid (GABA) transporter BGT1 (SLC6A12) accomplish cellular accumulation of organic osmolytes and thus contribute to cell volume regulation. Challenges of cell volume constancy include energy depletion, which compromises the function of the Na^+/K^+ ATPase leading to cellular Na^+ accumulation and subsequent cell swelling. Energy depletion is sensed by AMP-activated protein kinase (AMPK). The present study explored whether AMPK influences the activity of SMIT and BGT1. To this end, cRNA encoding SMIT or BGT1 was injected into *Xenopus* oocytes with and without additional injection of wild type AMPK (AMPKα1 + AMPKβ1 + AMPKγ1), of constitutively active $^{\gamma R70Q}$AMPK (AMPKα1 + AMPKβ1 + ^{R70Q}AMPKγ1) or of catalytically inactive $^{\alpha K45R}$AMPK (^{K45R}AMPKα1 + AMPKβ1 + AMPKγ1). Substrate-induced current in dual electrode voltage-clamp experiments was taken as measure of osmolyte transport. As a result, in SMIT-expressing, but not in water-injected *Xenopus* oocytes, myoinositol, added to the extracellular bath, generated a current (I_{SMIT}), which was half maximal (K_M) at ≈7.2 μM myoinositol concentration. Furthermore, in BGT1-expressing, but not in water-injected *Xenopus* oocytes, GABA added to the bath generated a current (I_{GABA}), which was half maximal (K_M) at ≈0.5 mM GABA concentration. Coexpression of AMPK and of $^{\gamma R70Q}$AMPK but not of $^{\alpha K45R}$AMPK significantly decreased I_{SMIT} and I_{GABA}. AMPK decreased the respective maximal currents without significantly modifying the respective K_M. In conclusion, the AMP-activated kinase AMPK is a powerful regulator of the organic osmolyte transporters SMIT and BGT1 and thus interacts with cell volume regulation.

1. Introduction

An obvious requirement for survival is the ability of the cell to limit alterations of cell volume [1]. Excessive cell swelling may eventually disrupt the cell membrane and thus cause necrotic cell death [1,2]. Conversely, excessive cell shrinkage may trigger suicidal cell death or apoptosis [1,3,4].

Cell volume regulatory mechanisms stimulated by cell swelling include release of ions through activation of K^+ channels and/or anion channels, KCl-cotransport, or parallel activation of K^+/H^+ exchange and Cl^-/HCO_3^- exchange [1,5,6] Cell volume regulatory mechanisms stimulated by cell shrinkage involve accumulation of ions through activation of Na^+,K^+,$2Cl^-$ cotransport, Na^+/H^+ exchange in parallel to Cl^-/HCO_3^- exchange, or Na^+ channels [1,7,8]. The Na^+ taken up during regulatory ion transport is extruded by the Na^+/K^+ ATPase in exchange for K^+. Following shrinkage cells further accumulate organic osmolytes, such as sorbitol, glycerophosphorylcholine and monomeric amino acids by altered metabolism as well as myoinositol (inositol), betaine, taurine and amino acids by Na^+-coupled transport [9–11]. Following swelling, cells release the organic osmolytes [9].

Myoinositol (inositol) is transported by the Na^+-coupled transporter SMIT (SLC5A3) and betaine/γ-aminobutyric acid (GABA) by the Na^+, Cl^--coupled transporter BGT1 (SLC6A12) [9,12–14]. Cell shrinkage stimulates the expression of the transporters and thus the cellular accumulation of the respective osmolytes [9,15]. The carriers may be sensitive to further cell stress conditions, such as radiation [16].

Challenges of cell volume constancy include cellular energy depletion, which impairs the function of the Na^+/K^+ ATPase, eventually leading to dissipation of the Na^+ and K^+ gradients, depolar-

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isation of the cell membrane, cellular accumulation of Cl^-, osmotic water entry and thus cell swelling [1]. Activation of mechanisms serving regulatory cell volume decrease and inhibition of mechanisms accomplishing regulatory cell volume increase may delay cell swelling and thus foster survival of energy-depleted cells. Accordingly, at least in theory, defence mechanisms against cell swelling during energy depletion may include inhibition of osmolyte transporters, such as SMIT and BGT1.

Energy depletion stimulates the AMP-activated protein kinase (AMPK), which senses the cytosolic AMP/ATP concentration ratio and thus the energy status of the cell [17,18]. AMPK-regulated functions include the stimulation of cellular glucose uptake, of glycolysis, of fatty acid oxidation and of enzymes required for ATP production [18–21]. The functions triggered by AMPK thus enhance the capacity of the cell to generate ATP [22]. AMPK further downregulates energy-utilising mechanisms including protein synthesis, gluconeogenesis and lipogenesis [18,19,22]. The AMPK-dependent regulation of metabolism confers protection against cell death during energy depletion [22–24]. To the best of our knowledge, nothing is known about an influence of AMPK on osmolyte transporters. The present study thus explored whether AMPK regulates the myoinositol transporter SMIT and/or the betaine/GABA transporter BGT1.

2. Materials and methods

2.1. Constructs

For generation of cRNA [25], constructs were used encoding wild type SMIT [26], wild type BGT1 [27], wild type AMPKα,β,γ [28], constitutively active $^{\gamma 1R70Q}$AMPK [29], and kinase dead mutant $^{\alpha K45R}$AMPK [30]. The cRNA was generated as described previously [31,32].

2.2. Voltage-clamp in Xenopus oocytes

Xenopus oocytes were prepared as previously described [33,34]. 4.6 ng of cRNA encoding either, AMPK (AMPKα1 + AMPKβ + AMPKγ), $^{\gamma R70Q}$AMPK (AMPKα1 + AMPKβ1 + ^{R70Q}AMPKγ1) or $^{\alpha K45R}$AMPK (^{K45R}AMPKα + AMPKβ + AMPKγ) were injected on the first (BGT1-experiments) or fourth (SMIT-experiments) day, 15 ng cRNA encoding SMIT and/or BGT1 on the first day after preparation of *Xenopus laevis* oocytes. The measurements were performed 3 days (BGT) or 7 days (SMIT) after the first injection. The oocytes were maintained at 17 °C in ND96-A solution containing 88.5 mM NaCl, 2 mM KCl, 1.8 mM $MgC1_2$, 0.1 mM $CaC1_2$, 5 mM HEPES, 0.11 mM tretracycline, 4 μM ciprofloxacin, 0.2 mM gentamycin (Refobacin©), 0.5 mM theophylline (Euphylong©) as well as 5 mM sodium pyruvate. The pH was adjusted to 7.4 by addition of NaOH. Two-electrode voltage-clamp recordings were performed at a holding potential of −90 mV. The data were filtered at 10 Hz, and recorded with a Digidata A/D–D/A converter and Chart V.4.2 software for data acquisition and analysis (Axon Instruments) [35]. The control solution (ND96) contained 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES. The final solutions were titrated to pH 7.4 using NaOH. γ-aminobutyric acid (GABA) was added to the solutions at a concentration of 2 mM unless otherwise stated. The flow rate of the superfusion was 20 ml/min, and a complete exchange of the bath solution was reached within about 10 s [36].

2.3. Statistical analysis

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

3. Results

3.1. AMPK downregulated SMIT-mediated electrogenic inositol transport

Electrogenic myoinositol transport was minimal in water-injected *Xenopus* oocytes (Fig. 1). In oocytes expressing SMIT, however, myoinositol (1 mM) induced an inward current (I_{SMIT}) reflecting electrogenic entry of Na^+ and myoinositol. I_{SMIT} was significantly decreased by additional expression of the AMP-activated protein kinase (AMPK = AMPKα1 + AMPKβ1 + AMPKγ1). Thus, AMPK downregulated SMIT activity. The effect of wild type AMPK was mimicked by constitutively active $^{\gamma R70Q}$AMPK (AMPKα1 + AMPKβ1 + ^{R70Q}AMPKγ1). I_{SMIT} tended to be lower in *Xenopus* oocytes coexpressing SMIT with $^{\gamma R70Q}$AMPK than in *Xenopus* oocytes coexpressing SMIT with wild type AMPK, a difference, however, not reaching statistical significance (Fig. 1). SMIT activity was not significantly modified by coexpression of the inactive $^{\alpha K45R}$AMPK (^{K45R}AMPKα + AMPKβ + AMPKγ). I_{SMIT} was not significantly different between *Xenopus* oocytes expressing SMIT together with $^{\alpha K45R}$AMPK and *Xenopus* oocytes expressing SMIT alone (Fig. 1).

A kinetics analysis was performed to explore, whether AMPK modifies SMIT-dependent myoinositol transport by influencing the maximal transport rate or by altering the affinity of the carrier. Kinetics of myoinositol-induced currents in SMIT-expressing *Xenopus* oocytes (Fig. 2) yielded a maximal current of 5.2 ± 0.2 nA. The myoinositol concentration needed for half maximal current (K_M) was 7.2 ± 1.4 μM ($n = 9$). The coexpression of constitutively active $^{\gamma R70Q}$AMPK did not significantly modify K_M (12.7 ± 3.9 μM), but

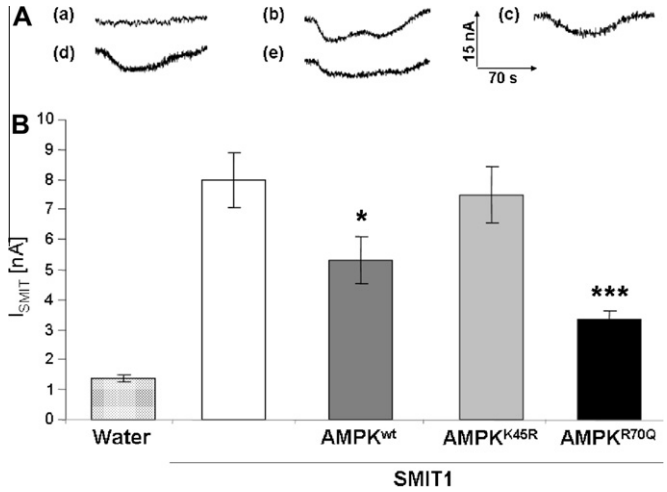

Fig. 1. Coexpression of wild type AMPK, or of constitutively active $^{\gamma R70Q}$AMPK but not of the dead mutant $^{\alpha K45R}$AMPK down-regulated electrogenic myoinositol transport in SMIT-expressing *Xenopus* oocytes. (A) Original tracings of the myoinositol-induced inward current (I_{SMIT}) in *Xenopus* oocytes injected with (a) water, expressing SMIT without (b) or with additional coexpression of (c) wild type AMPK [AMPKα1 + AMPKβ1 + AMPKγ1], of (d) inactive $^{\alpha K45R}$AMPK [^{K45R}AMPKα1 + AMPKβ1 + AMPKγ1] or of (e) constitutively active $^{\gamma R70Q}$AMPK [AMPKα1 + AMPKβ1 + ^{R70Q}AMPKγ1]. (B) Arithmetic means ± SEM of inositol- (1 mM) induced current (I_{SMIT}) in *Xenopus* oocytes injected with water (dotted bar, $n = 17$), expressing SMIT without (white bar, $n = 16$) or with additional coexpression of wild type AMPK (dark grey bar, $n = 16$), with inactive $^{\alpha K45R}$AMPK (light grey bar, $n = 12$) or with constitutively active $^{\gamma R70Q}$AMPK (black bar, $n = 16$). * ($p < 0.05$), *** ($p < 0.001$) indicate statistically significant difference from current in *Xenopus* oocytes expressing SMIT alone.

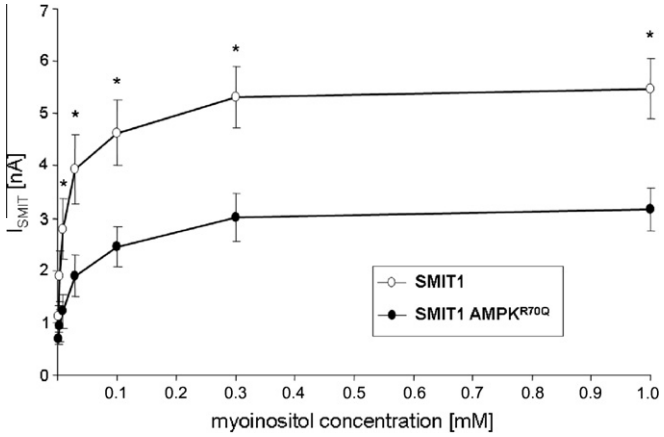

Fig. 2. AMPK decreased the maximal current of SMIT without appreciably affecting affinity. Arithmetic means ± SEM of I_{SMIT} as a function of the myoinositol concentration in *Xenopus* oocytes expressing SMIT without (open circles, $n = 9$) and with (closed circles, $n = 6$) constitutively active $^{\gamma R70Q}$AMPK * indicates statistically significant difference from *Xenopus* oocytes coexpressing $^{\gamma R70Q}$AMPK.

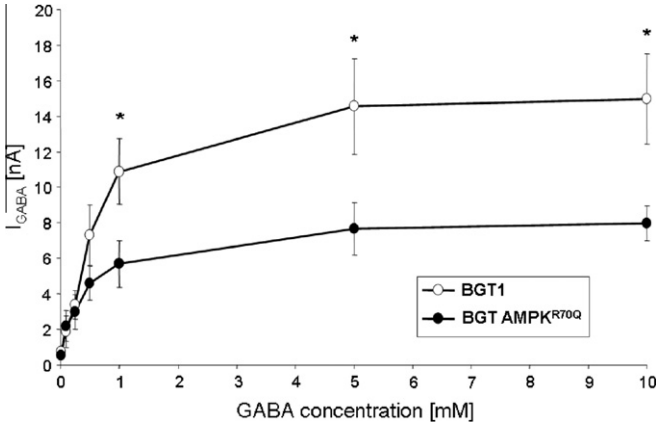

Fig. 4. AMPK decreased the maximal current of BGT1 without appreciably affecting affinity. Arithmetic means ± SEM of I_{GABA} as a function of GABA concentration in *Xenopus* oocytes expressing BGT1 without (open circles, $n = 6–9$) and with (closed circles, $n = 6–8$) constitutively active $^{\gamma R70Q}$AMPK. * Indicates statistically significant difference from *Xenopus* oocytes coexpressing $^{\gamma R70Q}$AMPK.

significantly ($p < 0.05$) decreased the maximal current (3.02 ± 0.17 nA, $n = 6$).

3.2. AMPK downregulated BGT1-mediated electrogenic GABA transport

GABA-induced currents were minimal in noninjected or water-injected *Xenopus* oocytes (Fig. 3). In *Xenopus* oocytes expressing BGT1, however, GABA (2 mM) induced an inward current (I_{GABA}) pointing to electrogenic transport of Na^+ and GABA. I_{GABA} was significantly decreased by additional expression of wild type AMPK (Fig. 3). The effect of wild type AMPK was mimicked by constitutively active $^{\gamma R70Q}$AMPK but not by the kinase dead mutant $^{\alpha K45R}$AMPK (Fig. 3). I_{GABA} was lower in BGT1- and $^{\gamma R70Q}$AMPK- or BGT- and wild type AMPK-expressing *Xenopus* oocytes than in *Xenopus* oocytes expressing BGT1 alone, whereas I_{GABA} was not significantly different between *Xenopus* oocytes expressing BGT1 together with $^{\alpha K45R}$AMPK and *Xenopus* oocytes expressing BGT1 alone (Fig. 3).

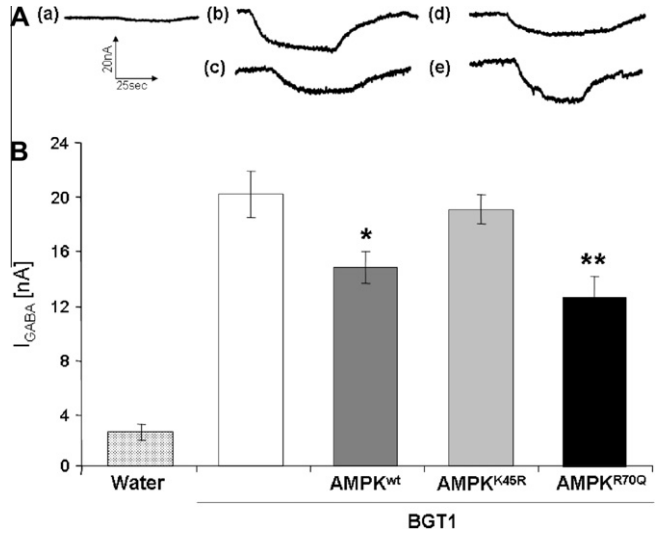

Fig. 3. Coexpression of wild type AMPK, or of constitutively active $^{\gamma R70Q}$AMPK but not of dead mutant $^{\alpha K45R}$AMPK down-regulated electrogenic GABA transport in BGT1-expressing *Xenopus* oocytes. (A) Original tracings of GABA-induced current recorded in *Xenopus* oocytes injected with (a) water, expressing BGT1 without (b) or with additional coexpression of (c) wild type AMPK [AMPKα1 + AMPKβ1 + AMPKγ1], of (d) constitutively active $^{\gamma R70Q}$AMPK [AMPKα1 + AMPKβ1 + ^{R70Q}AMPKγ1], or of (e) inactive $^{\alpha K45R}$AMPK [^{K45R}AMPKα1 + AMPKβ1 + AMPKγ1]. (B) Arithmetic means ± SEM of γ-aminobutyric acid- (GABA) (2 mM) induced current (I_{GABA}) in *Xenopus* oocytes injected with water (dotted bar, $n = 10$), expressing BGT1 without (white bar, $n = 15$) or with additional coexpression of wild type AMPK (dark grey bar, $n = 12$), with constitutively active $^{\gamma R70Q}$AMPK (black bar, $n = 14$), or with inactive $^{\alpha K45R}$AMPK (light grey bar, $n = 10$). * ($p < 0.05$), ** ($p < 0.01$) indicate statistically significant difference from current in *Xenopus* oocytes expressing BGT1 alone.

A kinetics analysis revealed that AMPK decreased BGT1 activity by decreasing the maximal transport rate without significantly modifying the affinity of the carrier (Fig. 4). In *Xenopus* oocytes expressing BGT1 alone, maximal I_{GABA} approached a value of 15.23 ± 0.48 nA ($n = 6$). The GABA concentration needed for half maximal I_{GABA} (K_M) was 0.53 ± 0.04 mM. The coexpression of constitutively active $^{\gamma R70Q}$AMPK did not significantly modify K_M (0.48 ± 0.08 mM), but significantly decreased the maximal I_{GABA} (8.87 ± 0.04 nA, $n = 6$).

4. Discussion

According to the present study, the AMP-dependent kinase AMPK downregulates both, the Na^+-coupled inositol transporter SMIT (SLC5A3) and the Na^+,Cl^--coupled betaine/γ-aminobutyric acid (GABA) transporter BGT1 (SLC6A12). The inhibition of the osmolyte transporters by AMPK during energy depletion avoids uptake of the respective organic osmolytes. Cellular Na^+, Cl^- and organic osmolyte uptake would otherwise drive the uptake of osmotically obliged water and would thus lead to cell swelling. The inhibition of SMIT is expected to decrease the cytosolic myo-inositol concentration and inhibition of BGT1 to decrease the cytosolic betaine and GABA concentration. Moreover, the decreased activity of either carrier curtails Na^+ and Cl^- entry into the cells. Accordingly, the inhibition of the carriers is expected to shrink the respective cells. The reduced osmolar load would counteract cell swelling following accumulation of Na^+ and Cl^- during energy depletion. Accordingly, the AMPK-dependent regulation of SMIT and BGT1 contributes to the protective effect of AMPK during energy depletion. In theory, AMPK may influence further cell volume regulatory transport systems, such as further osmolyte transporters and/or further cell volume regulatory transport proteins. In any case, the inhibition of SMIT and BGT1 participates in the defence against cell swelling during energy depletion.

In contrast to osmolyte transport, AMPK stimulates glucose uptake [18,19], an effect largely due to activation of the facilitative glucose carriers GLUT1, GLUT2, GLUT3 and GLUT4 [37–48]. In addition, AMPK activates the Na^+-coupled glucose transporter SGLT1 [49,50]. Similar to the osmolyte transporters, SGLT1 mediates Na^+ entry, requiring energy-consuming extrusion of Na^+ by the Na^+/K^+ ATPase [1]. The stimulation of SGLT1 by AMPK thus appears counterintuitive. However, in contrast to the osmolyte transporters, SGLT1 mediates the cellular accumulation of metabolic fuel. Degradation of glucose taken up by SGLT1 yields more ATP than needed to extrude the cotransported Na^+. AMPK further stimulates glycolysis, fatty acid oxidation and expression of enzymes required for ATP production [18,19]. Accordingly, AMPK counteracts ATP depletion by both, inhibition of ATP consumption and stimulation of ATP generation.

Stimulation of AMPK is not only observed following increase in the AMP/ATP ratio, but as well following an increase in cytosolic Ca^{2+} activity [17] or a decrease of O_2 levels [51]. Moreover, activation of AMP kinase has been observed following exposure of skeletal muscle cells to nitric oxide [52]. Thus, alterations of cytosolic Ca^{2+} activity, oxygen concentration and nitric oxide formation may influence SMIT- and BGT1-dependent cellular osmolyte uptake by regulating the activity of AMP kinase.

5. Conclusions

In conclusion, the AMP-dependent kinase AMPK decreases the activity of the Na^+-coupled myoinositol transporter SMIT and the Na^+, Cl^--coupled betaine/γ-aminobutyric acid (GABA) transporter BGT1. AMPK thus participates in the regulation of cell volume.

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