Tyrosine kinase inhibitors and immunosuppressants perturb the *myo*-inositol but not the betaine cotransporter in isotonic and hypertonic MDCK cells

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Tyrosine kinase inhibitors and immunosuppressants perturb the *myo*-inositol but not the betaine cotransporter in isotonic and hypertonic MDCK cells.

**Background.** The sodium/*myo*-inositol cotransporter (SMIT) and the betaine cotransporter (BGT1) are essential for the accumulation of *myo*-inositol and betaine, and hence cell survival in a hypertonic environment. The underlying molecular mechanism involves an increase in transcription of the SMIT and BGT1 genes through binding of a *trans*-acting factor to enhancer elements in the 5′ flanking region of both genes, resulting in increased mRNA abundance and increased activity of the cotransporters. Current evidence regarding transcriptional and post-transcriptional regulation indicates that both cotransporters are regulated in parallel.

**Methods.** To investigate the signal transduction of hypertonic stress, we examined the effect of tyrosine kinase inhibitors and immunosuppressants on the hypertonicity-induced activity of the two cotransporters in Madin-Darby canine kidney (MDCK) cells.

**Results.** None of the agents studied affected BGT1 activity in isotonic or hypertonic conditions. Treatment of MDCK cells with genistein, a tyrosine kinase inhibitor, increased SMIT activity in hypertonic but not isotonic conditions. The stimulation of SMIT by genistein was accompanied by a parallel increase in mRNA abundance. In contrast, treating cells with tyrphostin A23, another tyrosine kinase inhibitor, or cyclosporine A, an immunosuppressant, inhibited SMIT activity in hypertonic cells. FK506, another immunosuppressant, increased SMIT activity, but only in isotonic conditions.

**Conclusions.** These results provide the first evidence of divergent regulatory pathways modulating SMIT and BGT activity.

Constant cell volume is important for normal cell function and survival. Maintaining a constant cell volume during changes in ambient tonicity (effective osmolarity) is a critical problem for cells. Most cells respond to tonicity changes by activating specific metabolic and transport processes that restore cell volume to its normal resting state. These processes mediate the transport of inorganic ions, as well as small organic molecules known as compatible osmolytes [1–3]. In contrast to perturbing ions, for example, potassium and sodium, organic nonperturbing osmolytes have biophysical and biochemical properties that allow cells to withstand large shifts in their concentration without deleterious effects on cellular structure and function [4]. *Myo*-inositol and betaine are major organic nonperturbing osmolytes that accumulate to very high levels in hypertonic renal medulla [5]. Their accumulation is the result of an increase in the activity of their respective transporters, the Na+/*myo*-inositol cotransporter (SMIT) and the Na⁺,Cl⁻/betaine cotransporter (BGT1). The accumulation of *myo*-inositol and betaine in response to hypertonicity is slow and usually requires 18 to 24 hours for completion, as it results from increased transcription of the SMIT and BGT1 genes and an increase in abundance of mRNA for the cotransporters [6, 7]. This response to hypertonicity is regulated in part by the activation and binding of a transcription factor, tonicity element-binding protein (TonEBP), to enhancer elements in the 5′ flanking region of the SMIT and BGT1 genes [8–10]. The pathway that leads to the activation of TonEBP is not known. However, P38 kinase has been shown to be activated by hypertonicity, and its inhibition prevents the induction of BGT1 in MDCK cells [11]. Several other protein kinases are activated by hypertonicity as well, but their role in the accumulation of compatible osmolytes is not clear [11, 12]. Protein tyrosine phosphorylation is part of the early hypertonic signaling transduction pathway that results in the activation of the Na⁺/H⁺ antiporter within minutes [13, 14]. In muscle cells and fat cells, a tyrosine kinase pathway signals the translocation of the insulin-responsive glucose transporter (GLUT4) from intracellular vesicles to the plasma membrane in response to hypertonic shock [15].

**Key words:** hypertonicity, cell volume, osmolytes, cotransport, protein phosphorylation, transcription.

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kinase C results in rapid inhibition of both the myo-inositol and the betaine cotransporter [16].

In this article, we describe the effects of tyrosine kinase inhibitors and of immunosuppressants on the activity of SMIT and BGT1 in isotonic and in hypertonic cells. Although in previous studies the activity of SMIT and BGT1 always behaved in a similar fashion [16–18], only SMIT activity was affected by the various agents we tested.

**METHODS**

Genistein, tyrphostin A23, and epidermal growth factor (EGF) were purchased from CalBiochem (La Jolla, CA, USA). Genistin was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Cyclosporins and FK506 were gifts from Dr. William Briggs (Division of Nephrology, Johns Hopkins University School of Medicine, Baltimore, MD, USA). 3H-myoinositol and 32P dCTP were purchased from Amersham Life Science (Arlington Heights, IL, USA), and 14C-betaine was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). Coon’s modified F12 medium was purchased from Irvine Scientific (Santa Ana, CA, USA), and Dulbecco’s modified Eagle’s medium was from Mediatech, Inc. (Herndon, VA, USA).

**Cell culture**

Madin-Darby canine kidney cells obtained from the American Type Culture Collection (Rockville, MD, USA) were studied in passages 20 to 30 after arrival in our lab. Cells were grown as previously described [19] in a serum-free defined medium, a 50:50 mixture of Dulbecco’s modified Eagle’s medium with 1 g/liter NaCl, 10 mM CaCl2, 1.2 mM MgCl2, and 10 mM Tris-HEPES, pH 7.4. Hypertonic cells received uptake solution containing 200 mM raffinose. To determine transporter activity, uptake was assayed at 37°C in air by adding to each well 1 ml of uptake solution containing 10 μM myo-inositol and 10 μM betaine supplemented with 0.2 μCi/ml 3H inositol and 0.04 μCi/ml 14C betaine. Treatment with the agent or diluent was continued during the 20-minute uptake period. This period represents the initial rate of the one hour linear uptake. Uptake was ended by rinsing each well three times with ice-cold stop solution (160 mM choline chloride, 10 mM Tris-HEPES, pH 7.4). In hypertonic cells, uptake was ended with ice-cold stop solution containing 200 mM raffinose. Cells were extracted overnight at room temperature by the addition of 1 ml of 0.25 M sodium hydroxide to each well. Extracts were neutralized with 1 M hydrochloric acid (62.5 μl HCl for 250 μl of extract) and assayed for 3H, 14C, and protein concentration (protein assay kit; Bio-Rad Laboratories, Hercules, CA, USA).

Madin-Darby canine kidney cells were exposed to various amounts of each agent to determine optimal concentrations. One-hundred μM genistein, 100 μM tyrphostin, 100 ng/ml EGF, and 3 μM cyclosporine were associated with significant cell death likely caused by prolonged exposure. Therefore, only concentrations with no effect on protein yield were used in subsequent experiments.

Epidermal growth factor, genistein, and tyrphostin were prepared as 20 ng/μl, 5.4 μg/μl, and 3.8 μg/μl stock solutions in distilled water, 100% ethanol, and dimethyl sulfoxide, respectively. Following dilution, the total concentration of these diluents in the culture medium was 0.15%. Cyclosporine was prepared as 0.5 μg/μl stock solution in 100% ethanol. Following dilution, the total concentration of ethanol in the culture medium was 0.24%. FK506 was prepared as 1 μg/μl stock solution in phosphate-buffered saline (PBS). Following dilution, the total concentration of PBS in the culture medium was 0.082%.

We also examined whether the change in activity of the cotransporters was the result of a short-term exposure to the agents. When MDCK cells were incubated in isotonic or hypertonic medium for 18 to 22 hours and then exposed for 30 minutes to genistein, tyrphostin, cyclosporine, or FK506 just before uptake was assayed, there was no effect on SMIT or BGT1 activity (data not shown), indicating that the effects shown in Figures 1 and 2 after prolonged treatment with these agents were not the result of short-term effects on the cotransporters. Defined medium contains myo-inositol but not betaine. When 100 μM betaine was added to the culture medium, however, the effect of 30 μM genistein, 30 ng/ml EGF, or 1 μM cyclosporin on the transporters activity was unchanged (data not shown).
Fig. 1. (A) Effect of epidermal growth factor (EGF) and various inhibitors on sodium/myo-inositol cotransporter (SMIT) and the betaine cotransporter (BGT1) activity in Madin-Darby canine kidney (MDCK) cells in isotonic medium. The medium contained the vehicle or the test agent (30 μM genistein, N = 7; 30 ng/ml EGF, N = 8; 30 ng/ml EGF plus 30 μM genistein, N = 5; 30 μM tyrphostin A23, N = 5; 30 ng/ml EGF plus 30 μM tyrphostin A23, N = 5; 1 μM cyclosporine, N = 5; or 1 μM FK506, N = 5). Incubation was 20 hours. The percent changes in SMIT activity (◇) and BGT1 activity (◼) are shown. Results are the mean ± se. *P values of < 0.05 comparing the action of each agent on SMIT or BGT1 activity to control are shown. (B) Effect of EGF and various inhibitors on SMIT and BGT1 activity in MDCK cells in hypertonic medium. The symbols and concentrations are the same as in (A).

Fig. 2. (A) Effect of each agent on SMIT activity relative to that on BGT1 activity in isotonic medium. *P values of < 0.05 comparing the action of each agent on SMIT activity to that on BGT1 are shown. (B) Effect of each agent on SMIT activity relative to that on BGT1 activity in hypertonic medium. *P values of < 0.05 are shown. The effect of cyclosporine approached but did not reach statistical significance (P = 0.068).
Northern analysis

Confluent MDCK cells in isotonic or hypertonic medium were treated with 30 μM genistein or diluent (alcohol) for 18 hours. Total RNA was extracted with Trizol (Life Technologies, Inc., Gaithersburg, MD, USA). For Northern analysis, RNA (10 μg per lane) was separated on a 1% agarose formaldehyde gel and transferred overnight to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA). The blot was ultraviolet cross-linked and cut just above the 28S marker. Hybridization of the upper part of the blot was carried out at 65°C overnight with canine SMIT cDNA probe [19]. The lower part of the blot was separately hybridized with canine betaine cotransporter (BGT1) cDNA probe [20] under the same conditions. The blots were washed twice with 2 × SSC (0.15 M sodium chloride and 0.15 M sodium citrate) containing 0.1% sodium dodecyl sulfate at room temperature for five minutes and once with 0.5 × SSC containing 0.1% sodium dodecyl sulfate at 65°C for 30 minutes. Radioactivity was detected by Phosphorimage analysis (Molecular Dynamics, Sunnyvale, CA, USA).

Statistics

The analysis of the extracts was performed in duplicate for each well, and the mean uptake in three experimental or control wells was considered as a single experiment. Results are expressed in pmol/minute/mg protein. Statistical analyses for isotonic and hypertonic conditions were performed by comparing the effect of each agent on SMIT and BGT1 to their paired controls using one-sample two-tailed t-tests. Comparing the effect of each agent on SMIT with that on BGT1 was done using the Wilcoxon signed-rank test.

RESULTS

Transport studies

Tyrosine kinase inhibitors. As previously reported, overnight exposure of MDCK cells to hypertonic medium resulted in an approximately fivefold increase in the activity of SMIT and BGT1 [17, 18]. Overnight treatment with 30 μM genistein had no effect on the activity of either transporter in isotonic cells (Fig. 1A). In hypertonic cells, genistein increased the activity of SMIT by 47% (Fig. 1B). Overnight treatment with genistin, an inactive form of genistein [21], had no effect on SMIT or BGT1 activity in isotonic or hypertonic conditions. Because genistein is a recognized inhibitor of receptor tyrosine kinases, particularly epidermal growth factor receptor (EGFR) tyrosine kinase, we tested the effect of EGF on SMIT and BGT1. Isotonic cells treated with 30 ng/ml EGF showed a 30% increase in SMIT activity and little effect on BGT1 activity (Fig. 1A). Epidermal growth factor had no effect on SMIT or BGT1 activity in hypertonic cells. When we tested the effect of simultaneous addition of genistein and EGF, the increase in SMIT activity in isotonic cells, presumably the effect of EGF, was still evident, as was the increase in SMIT in hypertonic cells, presumably the effect of genistein (Fig. 1). The results in isotonic cells suggest that genistein is not acting by inhibiting tyrosine kinase activity of the EGF receptor (or that EGF is acting via a genistein insensitive receptor). Although further studies are required to define the site of action of EGF and genistein in these experiments, it is noteworthy that only the activity of SMIT was affected. The data are presented in Figure 2 as a ratio of the effect on SMIT to that on BGT1.

To further examine the role of tyrosine kinases in the regulation of SMIT and BGT1, we tested another tyrosine kinase inhibitor, tyrphostin A23. Like genistein, overnight treatment with 30 μM tyrphostin A23 had no effect on the activity of SMIT or BGT1 in isotonic cells (Fig. 1A). In hypertonic cells, however, incubation with tyrphostin A23 inhibited the activity of SMIT by 20% (Fig. 1B). BGT1 activity in the same cells was not affected (Fig. 1B). The selective effect on SMIT activity relative to that on BGT1 activity is shown in Figure 2B. As with genistein, the effect of tyrphostin A23 on SMIT activity was evident in hypertonic cells simultaneously treated with 30 μM tyrphostin A23 and 30 ng/ml EGF (Fig. 1B). However, unlike that seen in the presence of genistein, the effect of EGF on SMIT activity was no longer significant in isotonic cells (Fig. 1A). Although tyrphostin A23 in the presence of EGF decreased BGT1 activity by 11%, the effect on SMIT relative to that on BGT1 remained significant (Fig. 2B).

Immunosuppressants

Tonicity element-binding protein, the transcription factor that mediates the stimulation of transcription of SMIT and BGT1, shares sequence similarity with the NF-AT family of transcription factors (unpublished observation). Because the immunosuppressants cyclosporin and FK506 inhibit calcineurin, which is involved in the activation of NF-AT transcription factors, the effects of these agents on SMIT and BGT1 activity were determined. One μM cyclosporin inhibited SMIT activity in hypertonic cells without affecting BGT1 activity (Fig. 1B). There was no effect on either transporter in isotonic cells (Fig. 1A). When isotonic MDCK cells were treated with 1 μM FK506, the activities of SMIT and BGT1 were increased (Fig. 1A). However, the change in BGT1 activity was small, only 7%. As seen with the previous agents, the effect of FK506 on SMIT activity relative to that on BGT1 activity in isotonic cells was significantly greater than unity (Fig. 2A). FK506 had no effect on hypertonic cells. Because cyclosporine and FK506 did not have the same effect on cotransporter activity, it is
The striking sensitivity of SMIT to the various inhibitors in contrast to the minimal or no effect on BGT1 raises the question of whether SMIT was affected because its substrate myo-inositol was present in the medium throughout the prolonged incubation with the inhibitors, whereas the substrate for BGT1, betaine, was not. To answer this question, we repeated the experiments that showed the most striking divergence in effect on the two cotransporters (genistein, EGF, and cyclosporin) using a defined medium, which is betaine free or a medium supplemented with 100 μM betaine, similar to the concentration of myo-inositol in the medium. The results in both sets of experiments were similar (data not shown). Only the activity of SMIT was affected, as in Figure 1, despite the presence of betaine throughout the incubation.

**DISCUSSION**

The most striking finding in this study is the divergent effect of each agent on SMIT and BGT1 activity. Although transcription has been shown to be a major step that leads to the accumulation of organic osmolytes in response to hypertonicity, it is evident that the regulation is complex and subject to perturbation by a number of factors. Although protein kinases are activated rapidly by hypertonicity [13, 14], the role of protein phosphorylation in the regulation of organic osmolyte accumulation, a late response to hypertonicity, has not been extensively studied. In contrast to the sole effect of a variety of agents on SMIT activity in this study, it was previously shown that activation of protein kinase A or protein kinase C inhibits the activity of SMIT and BGT1 after 30 minutes [16]. The agents used in this study had no effect in that period (data not shown).

Genistein is recognized as a receptor protein tyrosine kinase (PTK) inhibitor, particularly EGF receptor tyrosine kinase, with little effect on other protein kinases, phosphatases, or phosphodiesterases [22–26]. In this study, prolonged (20 hour) incubation with genistein stimulated the hypertonic response of SMIT without eliciting change in the BGT1 response. Genistein did not alter the activity of either transporter in isotonic cells. The lack of effect of genistein on BGT1 suggests that a PTK or some other genistein-sensitive step is involved in the regulation of SMIT activity but not BGT1 activity in hypertonic cells. Genistein may act by inhibiting phosphorylation of SMIT. More likely, especially in view of the prolonged exposure to genistein in these experiments, it is possible that genistein inhibits the phosphorylation of other proteins, which subsequently results in increased SMIT activity. The parallel increase in SMIT activity and mRNA abundance suggests that genistein affects SMIT regulation at the level of transcription in hypertonic cells. The lack of an effect on SMIT transcription

**Northern analysis**

The largest effect observed was with genistein treatment. To examine whether the genistein induced changes in SMIT activity are associated with changes in mRNA abundance, Northern blot analysis was performed on RNA prepared from MDCK cells treated for 18 hours with 30 μM genistein using SMIT and BGT1 cDNA probes. Treatment with 30 μM genistein increased SMIT mRNA in hypertonic cells (Fig. 3). To focus on the effect of genistein on SMIT mRNA relative to that on BGT1 mRNA, we used BGT1 mRNA as a control for loading because there was no effect of genistein on BGT1 activity in the uptake studies (Fig. 1B). This was performed by separately hybridizing the lower part of a single blot with a canine BGT1 cDNA probe and the upper part with a canine SMIT probe. In parallel with the uptake data, genistein significantly increased SMIT mRNA by 39% in three independent experiments. In contrast to genistein, we were unable to detect differences in mRNA abundance with other agents. This may be a reflection of smaller changes in mRNA abundance as well as in SMIT activity with these agents, or it may indicate that the effects of the other agents are post-transcriptional.
with agents other than genistein makes transcription an unlikely step in their action, although the sensitivity of the assay may not be great enough to detect small changes in mRNA abundance. The failure of genistein to block the effect of EGF on SMIT activity in isotonic cells was unexpected and may indicate that EGF is acting on isotonic cells via a genistein insensitive receptor. Tyrphostin A23, another PTK inhibitor [27, 28], elicited an effect different from that of genistein on SMIT activity. Further work is needed to test whether PTK is directly involved in the regulation of the hypertonic response of SMIT and to identify the molecular mechanism of the putative PTK-dependent pathway.

To further explore the possible role of protein phosphorylation in the regulation of SMIT and BGT1, we examined the effects of cyclosporine and FK506. Inhibition of the nuclear import of NF-AT transcription factor is largely responsible for the immunosuppressive action of cyclosporine and FK506 [29]. This results from specific inhibition of calcineurin, which in response to certain signals is responsible for the dephosphorylation of NF-AT and its translocation to the nucleus, where it participates in the activation of early response genes [30]. Because the transcription factor TonEBP is a common activator of toxicity enhancer elements in the 5′ flanking region of the SMIT and BGT1 genes, disturbances in TonEBP are expected to affect the regulation of SMIT and BGT1 similarly. The activity of TonEBP increases in response to hypertonicity with a time course similar to that of transcription of the SMIT and BGT1 genes [8, 10]. Sequence analysis of TonEBP (unpublished observation) [31] revealed a stretch of approximately 160 amino acids near the N-terminus that displays significant similarity to the Rel-like DNA-binding domain of the NF-AT transcription factor family. The failure of cyclosporin and FK506 to cause a change in BGT1 activity and their different effects on SMIT activity indicate that their actions do not involve TonEBP but rather occur through post-transcriptional pathways. It should be noted that cyclosporine and FK506 are structurally unrelated, and differences between their effects have been described previously [32].

The data presented here provide the first evidence of differential regulation of SMIT and BGT1 activities, to our knowledge. The results lay the groundwork for future studies designed to explore the signaling events that mediate the hypertonic activation of these genes, as well as the regulation of the two transport systems. We are currently developing specific antibodies to SMIT and BGT1 that will be critical to our analysis of the two molecules. These reagents, in combination with monoclonal antibodies specific for phosphorylated amino acids, will be useful in defining the potential alternative regulatory pathways that modify the accumulation of different compatible osmolytes in isotonic cells and their response to a hypertonic signal.

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