Downregulation of SGK1 by nucleotides in renal tubular epithelial cells

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By our group (3, 30, 31) have shown that these cells express SGK1 message and have both P2R subtypes, P2X receptor (a ligand-gated ion channel) and P2Y receptor (a G protein-coupled receptor, GPCR). Therefore, the studies were performed to evaluate whether extracellular nucleotides regulate the expression or function of SGK1 in mIMCD-3 cells.

MATERIALS AND METHODS

Chemicals. Polyclonal antibody for SGK1 was purchased from Cell Signaling Technology (Beverly, MA); polyclonal antibodies for phosphorylated SGK1 (p-SGK1) at serine residue (Ser422) and at threonine residue (Thr256) and GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). ATP, 3′-O-(4-benzoyl)benzoyl-ATP (BzATP), α,β-methylene-ATP (α,β-MeATP), 2-methylthio-ATP (2-MeSATP), and uridine triphosphate (UTP), suramin, pyridoxal-5′-phosphate-6-(2-naphthylazo)-6′-nitro-4′,8′-disulfonate (PPADS), adenosine, and α-aldosterone were purchased from Sigma-Aldrich (St. Louis, MO). DMEM/F12 medium, phenol red-free DMEM-F-12, and charcoal/dextran-stripped fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA). Regular FBS was purchased from HyClone Laboratories (Logan, UT).

Cell culture. The mIMCD-3 cell line was obtained from American Type Culture Collection (Manassas, VA). Cells were incubated in DMEM/F12 medium containing 5% FBS at 37°C with 95% O2-5% CO2. Cells of 15–20 passages in a confluent monolayer were used. For the experiments with aldosterone, cells were first grown to confluent monolayers in normal culture medium and then switched to phenol red-free DMEM-F-12 plus 5% charcoal/dextran-stripped fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA). Regular FBS was purchased from HyClone Laboratories (Logan, UT).

Protein isolation. For the isolation of total cell lysates, cells were washed twice with ice-cold PBS and then in the presence of a buffer solution containing 10 mM sodium orthovanadate, 1% SDS, and 10 mM Tris-HCl (pH 7.5). Afterward, cells were sonicated for 15 s and spun at ~15,000 g for 5 min. The supernatant was collected into a clean tube for Western blotting. For the isolation of the cytosolic fraction and membrane fraction, cells were washed twice with ice-cold PBS and scraped into 0.9 ml of cold PBS containing protease inhibitor cocktail. Afterward, cells were sonicated for 15 s and spun at ~500 g for 15 min at 4°C. The nuclei and unbroken cells were discarded, and the supernatant was further spun at ~148,000 g for 1 h at 4°C. The supernatant and pellet, for the respective cytosolic and membrane fractions, were transferred into clean tubes and prepared for Western blotting.

Western blotting. The actual amount of protein sample from each testing group was measured via a fluorescence plate reader (FL 600; Bio-Tek Instruments, Winooski, VT), and the protein concentration was normalized. Equal amounts of protein were boiled in the presence of loading buffers and then separated on 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was incubated in the blocking solution containing 5% nonfat milk and then in
the Tris-buffered saline containing the primary antibody, SGK1 (1: 1,000), p-SGK1 (Ser^422) (1:500), p-SGK1 (Thr^268) (1:500), or GAPDH (1:800), at room temperature for 2 h. After the membrane was washed three times in Tris-buffered saline containing 0.2% Tween 20, it was incubated in the antibody solution containing the horseradish peroxidase-conjugated secondary antibody. Enhanced chemiluminescent reagents were used to detect the antibody-bound proteins on the membrane according to the manufacturer’s instructions (Amersham Biosciences). SGK kinase activity assay. A nonradioactive enzyme-linked immunosorbent assay (ELISA) kit for SGK1 kinase activity was purchased from Stressgen Bioreagents (EKS-430A; Ann Arbor, MI) and used to screen the activity of SGK1. The amount of protein sample from each testing group was measured, and the concentration was normalized. Equal amounts of cytosolic protein samples from control cells and from different pretreatment groups of cells were added to the appropriate wells of a microtiter plate that were precoated with the substrate of SGK1. The assay procedure was performed according to the manufacturer’s instructions. The absorbance of the color from each well was measured with an absorbance detector (AD 340S; Beckman Coulter) at a wavelength of 450 nm. The assay was run at least in triplicate. The subtraction of the background optical density (OD) reading was applied to all the sample OD readings. The kinase activity is expressed as the ratio of OD reading from each sample and the OD reading from 16 ng of purified active recombinant SGK kinase (provided in the assay kit).

Statistical analysis. Gel digitizing software UN-SCAN-IT 6.0.3 (Silk Scientific, Orem, UT) was used for the analysis of all immunoblots. The total pixel intensities with four-corner background correction were exported into Origin 6.0 (Microcal Software, Northampton, MA) for statistical analysis. Densitometric quantification of SGK1 bands was reported as a percentage of the density value versus its loading control of GAPDH density value for each treatment group and then normalized to that for the control group. The results are expressed as means ± SE. The data from each treatment group were compared with the data from the control group by using an unpaired Student’s t-test. When data were compared among the treatment groups and the control group, a one-way ANOVA was performed with three groups or more before a t-test was performed. Differences were considered statistically significant if P < 0.05.

RESULTS

SGK1 protein is expressed in mIMCD-3 cells and is downregulated in cells treated with ATPγS. SGK1 gene expression was previously demonstrated in the mIMCD-3 cell line by Northern analysis (3). We first investigated the protein expression of SGK1 with Western blotting. Using an SGK1 polyclonal antibody, we detected a single immunoband at ~55 kDa from the total cell lysates, total membrane, and cytosolic fraction, respectively (Fig. 1, A–C). The expression of SGK1 immunoband in cells pretreated with ATPγS (a more stable analog of ATP, at 100 µM for 30 min; same treatment throughout the study) was slightly reduced in total cell lysates (~4%; P = 0.03; n = 3), but there was a large reduction of SGK1 immunoreactivity in the total membrane (~40%; P = 0.004; n = 3) and in the cytosol (~60%; P = 0.007; n = 3) (Fig. 1E).

We used p-SGK1 polyclonal antibodies to further examine the protein expression, because previous studies indicated that Thr^268 and Ser^422 of SGK1 are involved in the regulation of the kinase activity (5, 11, 19). Figure 1D shows that in cells pretreated with ATPγS, cytosolic p-SGK1 (Ser^422) was decreased significantly compared with the control cells (~49%; P = 0.02; n = 4). A similar percentage of reduction was obtained with p-SGK1 at Ser^422 compared with the reduction of total cytosolic SGK1 (see Fig. 1E), indicating that both the inactive and active forms of the kinase can be affected by ATPγS. However, we were unable to detect an appreciable immunoband with p-SGK1 (Thr^268) in the cytosolic fractions of either control cells or cells pretreated by ATPγS (data not shown).

ATPγS-induced downregulation of cytosolic SGK1 protein is reversed by P2R antagonists. To verify that the downregulation of SGK1 by ATPγS was mediated by the activation of P2R, we applied suramin and PPNDs, antagonists of P2R, to cells 10 min before ATPγS treatment (all at 100 µM). The reduction of the cytosolic SGK1 protein by ATPγS in those cells was partially reversed (Fig. 2; ATPγS + suramin and ATPγS + PPNDs compared with ATPγS only, P < 0.002 and P < 0.004, respectively; compared with control, P = 0.05 and P = 0.04, respectively; n = 3 in each group). Suramin and PPNDs themselves had no significant effect on the expression of cytosolic SGK1 protein (P = 0.3 and P = 0.7, respectively; n = 3 in each group). Together, these data suggest that the ATPγS-induced decrease in cytosolic SGK1 protein was mediated by P2R.

Cytosolic SGK1 protein is downregulated by other nucleotides. To further test the involvement of activation of the P2R subtypes P2X and P2Y in the downregulation of SGK1 expression, we incubated cells with the nucleotides BzATP, α,β-MeATP, 2-MeSATP, and UTP individually (100 µM; 30 min). BzATP, α,β-MeATP, and UTP caused a decrease in the expression of cytosolic SGK1 (Fig. 3A), indicating that both P2X and P2Y receptor subtypes were involved in the regulation of SGK1. The functional potencies of nucleotides in reducing cytosolic SGK1 are in the order of ATPγS (~55%; P < 0.02) > UTP (~50%; P < 0.02) > BzATP (~32%; P < 0.05) > α,β-MeATP (~19%; P = 0.07) (Fig. 3B). However, 2-MeSATP did not decrease the expression of SGK1. It is possible that either the cells lacked specific 2-MeSATP-sensitive P2R subtypes or 2-MeSATP-activated P2R subtypes were not involved in the downregulation of SGK1.

In a previous study by our group (30) using this cell line, the P1 receptor (P1R) agonist adenosine could induce a small increase in the intracellular Ca^{2+} concentration. Since activation of P1R has been shown to inhibit ENaC (28), we examined whether activation of P1R could affect cytosolic SGK. In cells that were pretreated with the P1R agonist adenosine (100 µM; 30 min), a small reduction of cytosolic SGK was observed via Western blotting analysis (~10%; P = 0.04; n = 3) (Fig. 3, C and D). Since the reduction level of cytosolic SGK was greater in the activation of P2R than in the activation of P1R (45–60 vs. 10%), we postulate that the downregulation of SGK1 by P2R agonists in our study was mainly mediated by P2R.

We also looked at the effects of ATPγS-treated cells after 5- and 10-min treatment periods. The reduction of cytosolic SGK protein expression was seen only in the 5-min treatment group (~12%; P = 0.002; n = 3) (Fig. 3, C and D). We were unable to detect changes of SGK1 with 10-min ATPγS treatment under similar experimental settings. SGK1 kinase activity in the cell cytosol is reduced by ATPγS. The Western blotting data indicate that activation of purinergic receptors affects the cytosolic SGK1 protein. To evaluate whether the function of SGK1 was also affected by activation of P2R in the cytosol, we next examined SGK1
kinase activity via ELISA analysis. Figure 4A shows that the basal level of SGK1 kinase activity was decreased significantly in cells pretreated with ATPγS (100 μM; 30 min) (ATPγS, 1.00 ± 0.04 vs. control, 1.20 ± 0.03; P < 0.03; n = 3).

The specificity of the inhibition via activation of P2R was further confirmed by experiments in which cells were treated with P2R antagonists before ATPγS application. ATPγS-inhibited SGK1 kinase activity was reversed by the presence of either suramin or PPNDS (all at 100 μM) (Fig. 4A, ATPγS + suramin, 1.19 ± 0.12 and ATPγS + PPNDS, 1.19 ± 0.06 vs. control, 1.20 ± 0.03; not significantly different; n = 3 in each group).

Since it is well established that aldosterone upregulates SGK1 protein in the renal cells (3, 4, 10, 12, 14), we used aldosterone as a positive control in the ELISA analysis. In cells pretreated with aldosterone (1 μM; 20 h), a significant increase of SGK1 kinase activity was detected (Fig. 4B, aldosterone, 1.63 ± 0.07 vs. control, 1.29 ± 0.06; P < 0.02; n = 4). However, this increase was suppressed completely in cells that were also pretreated with ATPγS (Fig. 4B, aldosterone + ATPγS, 1.20 ± 0.04 vs. control, 1.29±0.06; not significantly different; n = 4). Together, these data suggest that functional SGK kinase can be downregulated by activation of P2R.

DISCUSSION

In the current study, we demonstrated for the first time to our knowledge that SGK1 could be downregulated by nucleotides in renal collecting duct epithelial cells (mIMCD-3), likely via the activation of P2R. Our findings show that 1) in ATPγS-pretreated cells, the cytosolic fraction of SGK1 protein was reduced between 45 and 60% (Figs. 1–3); 2) the reduction of cytosolic SGK1 protein by ATPγS was not observed in the presence of P2R antagonists (Fig. 2); 3) activation of P2X and P2Y receptors by other nucleotides could reduce the expression of cytosolic SGK1 20–50% (Fig. 3A); activation of P1R by adenosine could reduce the expression of cytosolic SGK1 10% (Fig. 3C); 4) the basal level of cytosolic SGK1 kinase activity was decreased significantly in ATPγS-pretreated cells but not in those treated with P2R antagonists (Fig. 4A); and 5) aldosterone-stimulated increases in cytosolic SGK1 kinase activity could be suppressed by ATPγS (Fig. 4B).
**Mechanism of downregulation of SGK1 by nucleotides.** SGK1 is activated by phosphorylation at both the transcriptional and posttranslational levels by many extracellular signals (11). SGK1 engages in the regulation of renal function, including Na\(^+\) retention and K\(^+\) elimination of the kidney, thereby contributing to body salt homeostasis and blood pressure (10, 26). Although the exact signaling cascade of activation of P2R-mediated downregulation of SGK1 remains to be explored, GPCR (i.e., the P2Y receptor)-mediated downstream components of the phosphatidylinositol pathway may be involved, and this conceivably permits modulation of ion transport activity.

Activation of GPCR stimulates a phospholipase C (PLC)-mediated signaling cascade that hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)). PIP\(_2\) is a precursor not only of inositol 1,4,5-trisphosphate (IP\(_3\); which triggers the release of Ca\(^{2+}\) from the endoplasmic reticulum) and diacylglycerol (which activates protein kinase C, PKC), but also of phosphatidylinositol 3,4,5-trisphosphate (PIP\(_3\)), which is produced by the action of phosphatidylinositol (PI) 3-kinase (22). Phosphorylation of SGK1 occurs through a cascade by PIP\(_2\)- and PIP\(_3\)-dependent protein kinases (PDK) (5, 10, 19). Therefore, activation of P2Y receptors by ATP\(_\gamma\)S in our cell model could stimulate PLC and enhance degradation of PIP\(_2\) by increasing the hydrolysis of membrane PIP\(_2\), which could downregulate the activity of SGK1. In addition, reduced conversion of PIP\(_2\) to PIP\(_3\) might negatively affect PDK and downstream SGK1, as well.

**Functional role of P2X receptors in downregulation of SGK1.** In the present study, ATP\(_\gamma\)S-induced downregulation of SGK1 was partially reversed by PPND\(_S\) (a potent antagonist for P2X\(_1\)) and suramin (a potent antagonist for P2X\(_1\), P2X\(_3\), P2Y\(_2\), P2Y\(_4\), and P2Y\(_{11}\)) (9, 18). In addition, BzATP (a potent agonist for P2X\(_4\), P2X\(_7\), and P2Y\(_{11}\)), \(\alpha,\beta\)-MeATP (a potent agonist for P2X\(_1\) and P2X\(_3\)), and UTP (a potent agonist for P2Y\(_2\) and P2Y\(_4\)) reduced the level of cytosolic SGK1 protein to a lesser extent. Although the specific P2R subtypes remain to be determined, the pharmacological profile of P2R agonists and antagonists suggests that both P2X and P2Y receptor subtypes are involved in the SGK1 regulation.

If the role of P2X receptors is to interact with intracellular G protein-mediated PLC and to increase plasma membrane PIP\(_2\) hydrolysis (i.e., upstream control of SGK1 and ENaC), then it may be that the role of P2X receptors is to facilitate the action of P2Y receptors. This conjecture is supported by the following: 1) plasma membrane PLC is Ca\(^{2+}\) dependent (actually all 4 subtypes of PLC require Ca\(^{2+}\) for catalytic function) (22), and activation of P2X receptors could provide a local influx of Ca\(^{2+}\) near the plasma membrane to enhance the activity of PLC; 2) activation of P2Y receptors may require an activation of P2X receptors concurrently, since a previous study by our group (30) demonstrated that ATP, an agonist for both P2X and P2Y receptors, failed to induce a significant increase in intracellular Ca\(^{2+}\) signal in the presence of P2X receptor antagonists, indicating that P2X receptor-mediated signaling pathways could interact with P2Y receptor-mediated and PLC-dependent signaling pathways.

**Physiological significance of downregulation of SGK1 by nucleotides.** Previous studies in A6 epithelial cells by Ma et al. (15) showed that activation of P2R by ATP\(_\gamma\)S in the isolated membrane patch via patch-clamp pipette blocks ENaC activity in the cell-attached patch, and the authors proposed the involvement of PLC-mediated phosphatidylinositol pathway. In their follow-up work using the excised inside-out patch technique, Ma et al. (16) further demonstrated that increased PIP\(_2\) hydrolysis accelerated ENaC activity rundown. This mechanism has been confirmed by other investigators (for review, see Ref. 20) and is consistent with our findings, since our data showed that activation of both P2X and P2Y receptors by nucleotides reduced the expression of cytosolic SGK1 signif-
significantly. Although we did not perform a direct study to access ENaC activity by ATPγS, it is conceivable that as a consequence of reduction of SGK1 in the cell, a downregulation of ENaC activity might be expected (4, 10, 14, 27).

Although inhibition of amiloride-sensitive Na⁺ transport by nucleotides has been demonstrated in many epithelial cells, including those in the kidney and lung (13), the various mechanisms remain controversial. For example, an earlier study (6) showed that inhibition of benzamil-sensitive Na⁺ current by activation of P2Y receptors in rabbit collecting duct cells requires PKC activity but does not require changes in intracellular Ca²⁺. Another study with mouse cortical collecting duct (12) seems to support this mechanism, since the inhibition of amiloride-sensitive current was mediated by activation of P2Y receptors and the inhibition occurred independently of an increase of intracellular Ca²⁺. In contrast, experiments in mouse IMCD cells (17) demonstrated that inhibition of amiloride-sensitive Na⁺ current can occur through activation of both P2X and P2Y receptors. A more recent report (7) suggested that neither an increase in intracellular Ca²⁺ nor an activation of PKC was responsible for the inhibition of ENaC by activation of P2Y receptors in mouse trachea. Thus other mechanisms different from changes in intracellular Ca²⁺ or the activation of PKC appear to modulate ENaC activity (8, 15, 16). Our data showing downregulation of SGK1 by nucleotides provide one plausible explanation for the observed inhibition of amiloride-sensitive Na⁺ transport in the above studies.
Physiological significance of downregulation of aldosterone-stimulated SGK1 by ATPγS. The action of aldosterone has been shown to control Na+ reabsorption in the distal nephron through regulation of ENaC activity, and this regulation is associated with SGK genes (3, 4, 11, 27). Our data demonstrate that aldosterone-stimulated SGK1 kinase activity could be suppressed by the activation of P2R. There is a growing appreciation for nontraditional regulatory mechanisms that regulate Na+ transport in the collecting duct. The renal tubular purinergic signaling is emerging as one such potential mechanism (13, 23, 25). Although the interaction between nucleotides and aldosterone on the regulation of SGK1 function requires further investigation, the current study supports the hypothesis that renal tubular purinergic signaling provides a “yin” mechanism to potentially inhibit Na+ transport, at least in part, via downregulation of SGK1 expression, in contrast to other “yang” mechanisms to upregulate Na+ transport in the collecting duct. Therefore, the current results suggest that P2R agonists have the therapeutic potential to treat hypertension especially stimulated by mineralocorticoid.

Redistribution of cellular SGK1 by ATPγS. SGK1 is found in the cell membrane and cytosol and has a rapid turnover rate with a half-life of ~30 min (1, 10). SGK1 protein in the total cell lysates was reduced only 4% compared with the reduced levels in the membrane (40%) and in the cytosol (up to 65%) in ATPγS-treated cells (Fig. 1A vs. B and C), suggesting that translocation of SGK1 occurs. It is possible that the downregulation of SGK1 by nucleotides involves the redistribution of SGK1 from the membrane and cytosol to the nucleus. This speculation is supported by previous studies (2, 10, 19). For instance, the subcellular translocation of SGK1 in mammary epithelial cells has been observed from cytoplasmic and perinuclear regions to the nucleus after stimulation with serum (2).

Downregulation of both active and nonactive forms of SGK1 by ATPγS. By using p-SGK1 antibodies against specific phosphorylation sites, we observed the reduction of cytosolic phosphorylated form of SGK1 at Ser242 in ATPγS-pretreated cells. Furthermore, the proportion of the reduction of p-SGK1 at Ser242 is nearly the same as the reduction in total SGK (~50 vs. 45–60%; Fig. 1E vs. Figs. 2B and 3B). It is plausible that activation of P2R by ATPγS downregulates both phosphorylated and nonphosphorylated forms of SGK1. Both Ser242 and Thr266 of SGK1 are considered as phosphorylation sites involved in the regulation of functional SGK kinase activity (10), because mutations of these two sites cause significant decreases in the SGK1 kinase activity (19). Therefore, the observed reduction of p-SGK1 at Ser242 by ATPγS should decrease functional cytosolic SGK1 activity and consequently contribute to reduction of ENaC activity. We could not detect an appreciable immunoband with p-SGK1 at Thr266 in the cytosolic fractions from control cells and from cells pretreated by ATPγS, and thus we were unable to determine a difference between treated and untreated cells. It is possible that either p-SGK1 (Thr266) antibody is not working well or SGK1 is not phosphorylated at the Thr266 position in our experimental conditions.

In summary, the present study has shown that extracellular nucleotides can downregulate the expression of cytosolic SGK1 protein and its activity in the renal epithelial cells. Given aldosterone’s known effect to stimulate ENaC activity, our data suggest that activation of purinergic signaling provides an additional mechanism for control of Na+ transport through downregulation of SGK1 in the renal collecting duct.

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REFERENCES


