Activation of Serum- and Glucocorticoid-induced Protein Kinase (Sgk) by Cyclic AMP and Insulin*

Sgk (serum- and glucocorticoid-induced protein kinase) is a serine/threonine-specific protein kinase that is transcriptionally regulated by serum, glucocorticoids, and mineralocorticoids. Sgk regulates the amiloride-sensitive sodium channel in kidney principal cells. Insulin and insulin-like growth factor-1 stimulate activity of Sgk by a mechanism mediated by protein phosphatidylinositide-dependent kinases (PDK)-1 and -2. In this study, we demonstrate that incubation of transfected cells with 8-(4-chlorophenylthio)-cAMP (8CPT-cAMP; 0.2 mM) led to a 2-fold activation of recombinant Sgk expressed in COS7 cells. Furthermore, the combination of insulin plus 8CPT-cAMP elicited a larger response than either agent alone. The effect of insulin was inhibited by wortmannin (100 nM), but not by the cyclic AMP-dependent protein kinase (PKA) inhibitor, H89 (10 μM). As expected, the effect of 8CPT-cAMP was completely blocked by H89. Surprisingly, the effect of 8CPT-cAMP was also inhibited by wortmannin, suggesting that phosphorylation of Sgk by PDK-1 and/or -2 is required for activation by 8CPT-cAMP. Mutational analysis led to similar conclusions. The Thr369→Ala mutant, lacking the PKA phosphorylation site, was activated by insulin but not 8CPT-cAMP. In contrast, the Ser422→Ala mutant, lacking a PDK-2 phosphorylation site, was inactive and resistant to activation by either insulin or 8CPT-cAMP. In summary, Sgk is subject to complex regulatory mechanisms. In addition to regulation at the level of gene expression, the enzymatic activity of Sgk is regulated by multiple protein kinases, including PKA, PDK-1, and PDK-2. Cross-talk among these signaling pathways may play an important role in the pathogenesis of the hypertension associated with hyperinsulinemia, obesity, and insulin resistance.

Serum- and glucocorticoid-induced protein kinase (Sgk) is a serine/threonine-specific protein kinase that regulates sodium absorption by the amiloride-sensitive sodium channel in kidney principal cells (1, 2). Transepithelial sodium transport is an essential physiological function regulated by multiple hormone systems, including aldosterone, vasopressin, and insulin. Recent evidence has suggested that Sgk is an important molecular target that integrates the multiple endocrine inputs regulating sodium transport. Expression of the Sgk gene is regulated at the transcriptional level by serum, glucocorticoids, and aldosterone, all of which up-regulate gene transcription (2, 3). Furthermore, insulin as well as insulin-like growth factors stimulate Sgk activity by a mechanism requiring the participation of phosphatidylinositol 3-kinase. Activation of phosphatidylinositol 3-kinase leads to activation of two downstream protein kinase activities: phosphoinositide-dependent kinases (PDK)-1 and -2. Phosphorylation of Thr256 and Ser422 by PDK-1 and -2, respectively, leads to activation of Sgk (4, 5).

Thr369 of Sgk is contained within a consensus sequence for phosphorylation by cyclic AMP-dependent protein kinase (PKA) (6, 7). Because vasopressin leads to activation of adenylate cyclase (8), we hypothesized that phosphorylation of Sgk by protein kinase A (PKA) might mediate the action of vasopressin to stimulate sodium transport. In the present study, we obtained evidence that cyclic AMP activates recombinant Sgk expressed in COS7 cells. Moreover, cyclic AMP-induced activation of Sgk is inhibited by H89, a PKA inhibitor, and also by mutation of the putative PKA phosphorylation site (Thr369→Ala). In addition, we obtained evidence that phosphorylation by PDK-1 and/or PDK-2 is required for the ability of cyclic AMP to activate Sgk. These data provide additional evidence elucidating the mechanisms whereby Sgk mediates endocrine regulation of sodium transport.

EXPERIMENTAL PROCEDURES

Expression of Recombinant Sgk—We searched GenBank™ and identified several expressed sequence tags corresponding to human Sgk. Clones 645185 and 42669 were obtained from Research Genetics (Huntsville, AL) and were found to contain the complete coding sequence of Sgk1. Clone 645185, which contains the complete coding sequence of the enzyme, was used in our experiments. We used polymerase chain reaction (KlenTaq, CLONTECH, Palo Alto, CA) to introduce both a strong Kozak consensus sequence at the translation start site and a Myc-epitope tag at the N terminus. The cDNA, flanked by an EcoRI site upstream and a NotI site downstream, was ligated into the TA Cloning pCR™ 2.1 plasmid (Invitrogen, Carlsbad, CA) and subcloned into the multiple cloning sites of pcDNA 3.1+ (Invitrogen), pCI-neo (Promega, Madison, WI), and pEGFP-C1 (CLONTECH). In addition, using a polymerase chain reaction-based method (Quick change Site-directed mutagenesis Kit; Stratagene, La Jolla, CA), we introduced the following three mutations into the coding sequence of Sgk: Thr369→Ala, Ser422→Ala, and Thr256→Ala. All the mutations were confirmed by automated DNA sequencing using reagents supplied in the DNA Sequencing Kit (PerkinElmer Life Sciences-Applied Biosystems, Foster City, CA).

COS7 cells were plated at a density of 1.5 × 106 cells/ml in six-well plates and cultured overnight in DMEM-low glucose (Life Technologies, Inc.) containing 10% fetal bovine serum (Life Technologies, Inc.). The following day, cells were transfected with expression plasmids using LipofectAMINE (Life Technologies, Inc.) following the manufacturer’s instructions. We used pCI-neo Myc-Sgk (600 ng/well) to express Sgk in...
of in vitro kinase activity, and in most of the 32P labeling studies, eGFP-C1 Myc-Sgk (600 ng/well) was used for the 32P labeling study of the phosphorylation of the Sgk-GFP fusion protein (Fig. 6B). Three hours after transfecting the cells, the transfection medium was diluted 1:1 with DMEM medium containing 20% fetal bovine serum. The next day, the medium was replaced with DMEM containing 10% fetal bovine serum plus antibiotics (penicillin, 100 units/ml and streptomycin, 100 μg/ml). Twelve hours later, the medium was replaced with serum-free medium (DMEM containing insulin-free bovine serum albumin (0.1%) plus antibiotics).

In Vitro Kinase Assay—Cells were incubated at 37 °C in serum-free medium in the presence or absence of human recombinant insulin (1 μM; Sigma) or 8-(4-chlorophenylthio)-cAMP (0.2 mM; SCPT-cAMP; Sigma). Insulin was added 20 min prior to the time of cell lysis; and SCPT-cAMP (9) was added 60 min prior to lysing the cells. In some experiments, we added various inhibitors 100 min prior to lysing the cells: wortmannin (100 nM) (5), H89 (10 μM), or okadaic acid (600 nM) (11). H89 was dissolved in DMEM while wortmannin and okadaic acid were dissolved in Me2SO. In each case, an equal volume of solvent was added to cell cultures lacking the inhibitor. Wortmannin and H89 were obtained from Calbiochem, while okadaic acid was supplied by Sigma.

The cell monolayers were then solubilized in solubilization buffer (300 μl/well) for 20 min at 4 °C: Tris, 50 mM, pH 7.8; NaCl, 300 mM; Triton X-100, 0.5%; protease inhibitors Complete (Roche Molecular Biochemicals) and phosphatase inhibitors (NaF, 100 mM; sodium pyrophosphate, 5 mM; sodium orthovanadate, 2 mM; EDTA, 5 mM). Extracts from two replicate wells were pooled, and an aliquot (100 μl) was analyzed by immunoblotting with rabbit anti-Myc antibody (Santa Cruz Biotechnology) to assess the expression of Myc-Sgk. A second aliquot (450 μl) was immunoprecipitated with anti-Myc antibody (Santa Cruz Biotechnology) for the immune complex kinase assay (12). Antibody was prebound to protein G-UltraLink (15 μl; Pierce) at 4 °C for 120 min. The immunobilized antibody was sedimented, washed, and incubated with cell extract overnight at 4 °C on a Rotating wheel. The immune complexes were sedimented and washed three times with solubilization buffer and twice with a kinase buffer (Tris, 20 mM, pH 7.4; MgCl2, 10 mM). Pellets were finally resuspended in kinase buffer containing ATP (5 mM), protein kinase A inhibitor (1 μM; Sigma), dithiothreitol (1 mM), [γ-32P]ATP (0.02 mCi/sample), and Arg-Pro-Arg-Thr-Ser-Thr-Phe peptide substrate (1 mM) (5). The reaction was allowed to occur for 30 min at room temperature, with gentle agitation, prior to stopping with 10 μl of stopping solution (ATP, 1 mM; bovine serum albumin, 1%; HCl, 0.6% w/v). The reaction mix was centrifuged in an Eppendorf microcentrifuge for 10 min at 14,000 rpm. Supernatants (20 μl) were applied to a 2.1-cm Phosphorimage (300 μl/well) for 20 min at 4 °C. The gels were dried and exposed to film for 30 s (B, upper gel) or 5 min (B, lower gel). In the immunoblots, several different bands with different mobilities were detected. As described under “Results,” B1 appeared not to be phosphorylated, B2 was phosphorylated at relatively low stoichiometry, and B3 was phosphorylated at high stoichiometry.

The amino acid sequence of Sgk contains a potential protein kinase A phosphorylation site: Lys-Lys-Ile-Thr-Pro (amino acid residues 366–370). Therefore, we investigated the effects of cAMP upon Myc-Sgk activity. After transiently expressing Myc-Sgk in COS7 cells, the transfected cells were incubated in the presence or absence of cAMP, insulin, or both. Subsequently, the cells were lysed in detergent, Myc-Sgk was immunoprecipitated with anti-Myc antibody, and Sgk activity was assayed in immune complexes.

Incubation of cells in the presence of 8CPT-cAMP (0.2 mM) for 60 min led to a 2-fold activation of Myc-Sgk (Fig. 2A, column 7, lane 3). As expected, incubation with insulin for 20 min increased Myc-Sgk activity by 3-fold (p = 0.0002; Fig. 1A, lane 2). Since neither insulin nor cAMP affected the quantity of Myc-Sgk contained in the immunoprecipitates (Fig. 1B, lanes 2 and 3), we conclude that cAMP and insulin increased the specific activity of the enzyme.

H89 (10 μM), an inhibitor of protein kinase A, blocked the effect of SCPT-cAMP (Fig. 2A, column 7), but did not interfere with the ability of insulin to stimulate the kinase activity of Myc-Sgk (Fig. 2A, column 6). As shown previously (4, 5), wortmannin (100 nM) inhibited the effect of insulin to activate Sgk (Fig. 2B, column 6). However, unexpectedly, wortmannin also inhibited the ability of SCPT-cAMP to activate Myc-Sgk (Fig. 2B, column 7).
and inhibited the combined effect of the insulin plus 8CPT-cAMP (Fig. 2B, column 7).}

**Mutational Analysis of Phosphorylation Sites**—We carried out mutational studies to investigate the role of the various phosphorylation sites. Thr$^{369}$ → Ala mutant Myc-Sgk, which lacks the predicted phosphorylation site for protein kinase A, is unable to undergo activation in response to 8CPT-cAMP ($p = 0.39$; Fig. 3, column 7). This observation suggests that phosphorylation of Thr$^{369}$, presumably catalyzed by protein kinase A, mediates activation of Sgk in response to cAMP. In contrast, Thr$^{369}$ → Ala mutant Myc-Sgk, which lacks the predicted phosphorylation site for protein kinase A, is unable to undergo activation in response to 8CPT-cAMP ($p = 0.0002$; Fig. 3, column 6). This result is consistent with the observations reported above, demonstrating that wortmannin inhibits activation of Myc-Sgk in response to either 8-CPT-cAMP or insulin (Fig. 2). Thus, phosphorylation by PKA is not sufficient to activate Myc-Sgk; phosphorylation of Ser$^{422}$ and Thr$^{256}$, presumably by PDK2 and PDK1, is also required.

**Multiple Species of Sgk**—Using immunoblotting to analyze solubilized extracts from transfected cells expressing Myc-Sgk, we detected two major bands corresponding to recombinant Myc-Sgk: a dark $M_r$ 51,000 band and a light $M_r$ 49,000 band, designated B2 and B1, respectively (Fig. 1B, upper panel, lane 1). Insulin did not alter the relative intensities of B1 and B2 compared with untreated samples (Fig. 1B, upper panel, lane 2). In contrast, 8CPT-cAMP increased the intensity of B1 when added alone (Fig. 1B, upper panel, lane 3) or in combination with insulin (Fig. 1B, upper panel, lane 4). H89 and wortmannin do not inhibit the ability of 8CPT-cAMP to increase the intensity of the faster migrating band, B1 (Fig. 5A, lanes 4 and 8). Likewise, this effect was also observed with the two mutant forms of Myc-Sgk: Thr$^{369}$ → Ala and Ser$^{422}$ → Ala (Fig. 5B, lanes 2–4). Thus, the shift in electrophoretic mobility does not appear to require phosphorylation by PKA or PDK-2 and does not seem to be related to the activation of the enzymatic activity of Sgk.

When films were overexposed, additional weak bands of...
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Under these conditions, the fastest migrating species that the B2 and B3 bands corresponded to different forms (protein (Fig. 6). By fusing Sgk to the GFP moiety, we shifted both bands to higher molecular mass (Fig. 6, lanes 4 and 5). This demonstrates that both bands are forms of the Myc-Sgk molecule, rather than substrates for phosphorylation by Sgk.

**Effects of Okadaic Acid**—Incubation of cells in the presence of okadaic acid, an inhibitor of protein phosphatases 1 and 2a, led to a 10-fold increase in the activity of Myc-Sgk (Fig. 7, column 1 versus column 5). Indeed, the effect of okadaic acid was greater than the effects of either insulin or 8CPT-cAMP. Nevertheless, both agents were able to further increase Sgk activity even in the presence of okadaic acid (600 nM; p = 0.033 (insulin) and p = 0.039 (8CPT-cAMP). When the phosphorylation sites for PDK-2 (Ser422Ala-mutant; Fig. 8, columns 9–16) and PDK-2 (Thr256 → Ala mutant; data not shown) were abolished, this blocked the ability of okadaic acid to activate Myc-Sgk. In contrast, abolishing the protein kinase A phosphorylation site (Thr369 → Ala mutant; Fig. 9, lanes 13–16) did not impair the ability of okadaic acid to activate Myc-Sgk.

Okadaic acid (600 nM for 70 min) resulted in the appearance of the hyperphosphorylated B3 band (Fig. 6A, right panels, lane 5), consistent with Sgk kinase activation. Interestingly, like 8CPT-cAMP, okadaic acid increased the relative intensity of B1 (i.e. the B1:B2 ratio; Fig. 6A, left panels, lanes 1, 2, and 5). The observation was confirmed in separate experiments, where okadaic acid was added alone or in combination with insulin and 8CPT-cAMP (Fig. 5B, lanes 2–9).

**DISCUSSION**

**Regulation of Sgk**—The amiloride-sensitive epithelial sodium channel (ENaC) in principal cells of the kidney plays a central role in determining sodium balance. Because this channel is responsible for reabsorbing the majority of the sodium in the glomerular filtrate, even small changes in activity of the channel can have large effects upon the quantity of sodium excreted in the urine. Keeping with its important physiological role, the activity of ENaC is highly regulated. It has recently been demonstrated that Sgk activates ENaC. Moreover, Sgk itself is highly regulated. For example, transcription of the Sgk gene has been reported to be up-regulated by both mineralocorticoids and glucocorticoids (2, 3) and also by follicle-stimulating hormone (14, 15). In addition, Sgk can be phosphorylated on Thr256 and Ser422 by PDK-1 and -2, respectively; these phosphorylations mediate the ability of insulin, insulin-like growth factor-1, and serum to activate Sgk (4, 5). It has been proposed that these two phosphorylations may occur in a sequential fashion (5). First, insulin stimulates PDK-2 to phosphorylate Ser422, and this renders Sgk a better substrate for PDK-1, which phosphorylates Thr256. It is the phosphorylation of Thr256 by PDK-1 that is believed to be responsible for activation of Sgk (5).

In this publication, we demonstrate that cAMP can activate Sgk and that this effect is mediated by PKA, which directly phosphorylates Thr369 in Sgk. This hypothesis is supported by two principal lines of evidence: (a) activation of Sgk by 8CPT-cAMP is inhibited by H89, an inhibitor of PKA activity; and (b) substitution of Ala for Thr369 abolishes the PKA phosphorylation site in Sgk and also abolishes the ability of 8CPT-cAMP to activate Sgk. Taken together, these observations demonstrate that phosphorylation by PKA is required for activation of Sgk in response to 8CPT-cAMP. However, two observations suggest that phosphorylation of Thr369 is not sufficient to activate Sgk: (a) wortmannin markedly inhibits the ability of 8CPT-cAMP to activate Sgk; and (b) mutation of the PDK-1 and PDK-2 phosphorylation sites (Thr256 and Ser422 respectively) prevents activation of Sgk in response to 8CPT-cAMP. Although the requirement for multisite phosphorylation has not yet been fully explained, we would like to propose one possible mechanism. When PKA phosphorylates Thr369, this might make Sgk a better substrate for phosphorylation of Thr256 and Ser422 by PDK-1 and PDK-2, respectively. According to this hypothesis,
Phosphorylation of Thr\textsuperscript{369} renders Sgk such a good substrate that it can be phosphorylated by the basal activities of PDK-1 and PDK-2 without the need for insulin to activate those two enzymes. The resulting phosphorylation of Thr\textsuperscript{256} and Ser\textsuperscript{422} is sufficient to activate Sgk (4–6).

In contrast, although phosphorylation of Thr\textsuperscript{256} and Ser\textsuperscript{422} is required for insulin-stimulated activation of Sgk, PKA-mediated phosphorylation of Thr\textsuperscript{369} is not required for activation in response to insulin. Furthermore, Ser\textsuperscript{422} is required for activation of Sgk in response to okadaic acid, whereas Thr\textsuperscript{369} is not required. These observations suggest that phosphorylation by PDK-1 and/or PDK-2 are the principal protein kinases responsible for activating Sgk. Okadaic acid, an inhibitor of protein phosphatases 1 and 2a, also leads to activation of Myc-Sgk.

Taken together, these observations suggest that either protein phosphatase 1 or 2a is responsible for dephosphorylating Thr\textsuperscript{256} and Ser\textsuperscript{422}, thereby inactivating Sgk.

After this work was completed, Gonzalez-Robayna et al. (15)
indicated, okadaic acid (600 nM) was added. Sgk activity was calculated in experiments similar to those described in the legend to Fig. 1. Where calculated by subtracting the background incorporation of 32P catalyzed experiment.

are means ± S.E. obtained from two individual points in one experiment.

Fig. 8. The PDK2 phosphorylation site in Sgk is required for activation by okadaic acid. COS7 cells were transfected with expression plasmids for either Myc-Sgk or the Ser422 → Ala mutant Myc-Sgk in experiments similar to those described in the legend to Fig. 1. Where indicated, okadaic acid (600 nM) was added. Sgk activity was calculated by subtracting the background incorporation of 32P catalyzed by immunoprecipitates of extracts from nontransfected cells. Data are expressed as a fraction of the mean insulin-stimulated wild type Myc-Sgk. Data are means ± S.E. obtained from two individual points in one experiment.

Fig. 9. The PKA phosphorylation site is not required for activation of Sgk by okadaic acid. COS7 cells were transfected with expression plasmids for either Myc-Sgk or the Thr369 → Ala mutant Myc-Sgk in experiments similar to those described in the legend to Fig. 1. Where indicated, okadaic acid (600 nM) was added. Sgk activity was calculated by subtracting the background incorporation of 32P catalyzed by immunoprecipitates of extracts from nontransfected cells. Data are expressed as a percentage fraction of the mean insulin-stimulated wild type Myc-Sgk. Data are means ± S.E. obtained from seven individual points in three replicate experiments.

reported that protein kinase A contributed to mediating the action of follicle-stimulating hormone to increase Sgk activity in ovarian granulosa cells. They particularly emphasized the role of cAMP in inducing transcription of the Sgk gene. Moreover, they concluded that cAMP triggered a pathway that activated phosphatidylinositol 3-kinase. Nevertheless, these mechanisms are not sufficient to account for our observations. Our mutational studies provide strong direct evidence that activation in response to cAMP requires direct phosphorylation of Sgk by protein kinase A inasmuch as mutation of the protein kinase A phosphorylation site abolishes the response. In contrast, the same mutation does not impair the ability of the enzyme to be activated in response to insulin, a response that is mediated by PKD-1 and -2 but that does not require Thr369 in Sgk by protein kinase A.

Multiple Species of Sgk—When Myc-Sgk was analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting with anti-Myc antibody, we detected two closely spaced bands with Mr of 49,000 and 51,000 (designated as B1 and B2, respectively). In addition, when the blots were overexposed, we detected a third lower mobility band (designated B3) in extracts of cells that were stimulated with insulin and/or 8CPT-cAMP. Interestingly, 8CPT-cAMP increased the intensity of B1 (i.e. the highest mobility band). The alteration in electrophoretic mobility of B1 is likely due to a covalent modification of the protein. While the nature of this covalent modification is unknown, 32P labeling experiments carried out in intact cells suggest that B1 is not phosphorylated. Rather, it is possible that 8CPT-cAMP triggers a proteolytic cleavage that accounts for the decrease in apparent molecular mass. Several lines of evidence suggest that this 8CPT-cAMP-induced gel shift is not related to Sgk activation. For example, the mobility shift is observed with both Ser422 → Ala- and Thr369 → Ala-Myc-Sgk although these mutant forms of the enzyme are resistant to activation by 8CPT-cAMP. Furthermore, even at low concentrations (0.05-0.10 mM), which did not activate Myc-Sgk, 8CPT-cAMP increased the intensity of the B1 band (data not shown).

In contrast, B2 and B3 are both phosphorylated forms of Sgk. However, by comparing the immunoblots with the autoradiographs of 32P-labeled Myc-Sgk, we conclude that the stoichiometry of phosphorylation is higher for B3 than for B2. It is likely that the hyperphosphorylation accounts for the decrease electrophoretic mobility of B3. Furthermore, the intensity of B3 is increased in response to either insulin and/or 8CPT-cAMP, which correlates with activation of the enzyme.

In the immunoblotting experiments (Fig. 6A), band B3 is considerably weaker than band B2, suggesting that only a small percentage of Myc-Sgk molecules become hyperphosphorylated. If B3 represents the activated species, this observation suggests that a relatively small minority of Myc-Sgk molecules are responsible for the majority of Sgk activity. It is unclear why such a small percentage of Sgk molecules become hyperphosphorylated in response to insulin and/or cAMP.

Significance of cAMP-induced Activation of Sgk—cAMP functions as the second messenger for multiple signaling pathways. Thus, the existence of a PKA phosphorylation site in Sgk provides opportunities for regulation in many tissues. Three distinct isoforms of Sgk have been reported. While Sgk1 and Sgk3 are widely expressed in many tissues, Sgk2 has a more limited pattern of expression, i.e. liver, kidney, pancreas, and brain (6). Interestingly, while the protein kinase A phosphorylation site is conserved in both Sgk1 and Sgk2, it does not appear to be present in the sequence of Sgk3. In contrast, the phosphorylation sites for phosphoinositide kinases are conserved in all three isoforms.

In the kidney, activation of Sgk by PKA may mediate the ability of vasopressin to stimulate sodium transport (16). Interestingly, it has recently been reported that wortmannin...
inhibits vasopressin-induced sodium transport (17), a result in agreement with our findings. Furthermore, there is evidence that cAMP exerts regulatory influences upon Sgk in extra-renal tissues. For example, it has been reported that follicle-stimulating hormone activates Sgk in the ovary (14, 15). In addition to the role in normal physiology, it is possible that Sgk may play important roles in the pathophysiology of various disease states.

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