SGK1 Phosphorylation of IκB Kinase α and p300 Up-regulates NF-κB Activity and Increases N-Methyl-d-aspartate Receptor NR2A and NR2B Expression*§

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Serum- and glucocorticoid-inducible kinase 1 (SGK1) is a downstream target of phosphatidylinositol 3-kinase signaling, and it regulates various cellular and physiological functions, but the SGK1 substrate proteins and genes regulated by SGK1 are less known. Here we have identified IκB kinase α (IKKα) as a novel substrate of SGK1 by using biochemical and bioinformatic approaches. SGK1 directly phosphorylates IKKα at Thr-23 and indirectly activates IKKα at Ser-180. Furthermore, SGK1 enhanced nuclear factor κB (NF-κB) activity and up-regulated N-methyl-d-aspartate receptor NR2A and NR2B expression through activation of IKKα at Thr-23 and Ser-180, and these two residues play an equally important role in mediating these effects of SGK1. Although SGK1 does not phosphorylate IKKβ, IKKβ activity is still required for IKK complex activation and for SGK1 phosphorylation and activation of NF-κB. In addition, SGK1 increased the acetylation of NF-κB through phosphorylation of p300 at Ser-1834, and this also leads to NF-κB activation and NR2A and NR2B expression. Moreover, an endogenous stimulus of SGK1, insulin, increased IKKα and NF-κB phosphorylation as well as NF-κB acetylation and NF-κB activity, but SGK1 small interfering RNA transfection blocked these effects of insulin. In examination of the functional significance of the SGK1-IKKα-NF-κB signaling pathway, we found that transfection of the IKKα double mutant (IKKαT23A/S180A) to rat hippocampus antagonized SGK1-mediated spatial memory facilitation. Our results together demonstrated novel substrate proteins of SGK1 and novel SGK1 signaling pathways. Activation of these signaling pathways enhances NR2A and NR2B expression that is implicated in neuronal plasticity.

Serum and glucocorticoid-inducible kinase 1 (SGK1) is a member of the serine/threonine protein kinase family that is transcriptionally induced by serum and glucocorticoids (1). SGK1 is known to regulate a variety of cellular functions, including salt homeostasis, ion channel conductance, cell proliferation, and neuronal excitability (2). In addition, SGK1 promotes cell survival and regulates cell cycle progression through phosphorylation of the forkhead transcription factor FKHL1 (3, 4). More recently, SGK1 was found to modulate the excitatory amino acid transporter function through phosphorylation of Nedd4-2 (5). SGK1 is also known to implicate several physiological functions. For example, sgk1 mRNA expression is increased in animal models of Parkinson disease, suggesting a role of SGK1 in neuroprotection (6). SGK1 increases glutamate-induced current partly by increasing GluR6 protein level in plasma membrane of Xenopus oocytes expressing rat GluR6 (7). SGK1 was also found to increase neurite outgrowth in hippocampal neurons (8, 9). Moreover, SGK1 facilitates long term potentiation and spatial learning in rats (10).

SGK1 is a downstream target of phosphatidylinositol 3-kinase signaling (11). SGK1 is first phosphorylated at Ser-422 by 3-phosphoinositide-dependent PDK2, which enables SGK1 to be further phosphorylated at Thr-256 by PDK1 (12). In addition, SGK1 also receives upstream signals from cyclic AMP (13), extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) (14), p38 MAPK (15), and big mitogen-activated protein kinase 1 (16). However, with the biological functions and upstream signals of SGK1 characterized to a certain extent, the downstream targets of SGK1 are relatively less known. Because SGK1 phosphorylates substrate proteins that contain the RXRXX(S/T) motif, where X stands for any amino acid (12), in this study we investigated the SGK1 substrate and signaling pathway by using the phospho-(p)-motif antibody as a tool. We also examined the expression of genes that are regulated by this signaling pathway. Our results revealed that IκB kinase α (IKKα), but not IKKβ, is a novel substrate of SGK1 and that SGK1 phosphorylation of IKKα increases nuclear factor κB (NF-κB) activity and up-regulates the expression of the N-methyl-d-aspartate (NMDA) receptor subunit NR2A and NR2B. In addition, SGK1 also phosphorylates p300 directly, and SGK1 phosphorylation of p300 increases NF-κB activity and NR2A and NR2B expression.
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through enhanced acetylation of NF-κB. These results suggest that SGK1 phosphorylation of IKKα and p300 regulates neuronal plasticity.

**EXPERIMENTAL PROCEDURES**

Cell Culture Preparation, DNA Transfection, and Plasmid Construction—HEK293T were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. PC12 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% equine serum and 5% fetal calf serum and incubated at 37 °C in a humidified atmosphere with 5% CO₂. Transfection was made by using the Lipofectamine™ 2000 reagent (Invitrogen) in 12-well culture plates according to the manufacturer’s instructions. For NF-κB reporter assay, PC12 cells were maintained in the medium for 24 h after transfection and then maintained in serum-free medium for 18 h. After serum starvation, PC12 cells were changed to normal culture medium for 6 h with serum stimulation. Cell lysate was then collected by using passive lysis buffer (Promega, WI) and was ready for the next step.

Animals were killed by decapitation, and their hippocampal tissue was dissected out. Rat hippocampal tissue was lysed by brief sonication in lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1% IGEPA. CS–630, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 µg/ml pepstatin A, 20 µg/ml leupeptin, 20 µg/ml aprotinin, 50 mM NaF, and 1 mM Na₃VO₄. The HEK293T cell lysate was prepared in 1 ml of lysis buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM MgCl₂, 1% IGEPA. CS–630, 10% glycerol, 1 mM diethiothreitol (DTT), 50 mM β-glycerophosphate, 50 mM NaF, 10 µg/ml PMSF, 4 µg/ml aprotinin, 4 µg/ml leupeptin, and 4 µg/ml pepstatin.

**Immunoprecipitation (IP) and Western Blot Analysis**—For IP reaction, the specific primary antibodies were unconjugated to agarose beads, and rabbit or mouse IgG was used in the control group. For IP assay of SGK1 and IKKα, the clarified hippocampal lysate (1 mg) was immunoprecipitated with 4 µl of anti-phospho-Thr-256-SGK1 antibody (Upstate Biotechnology, Inc.) or anti-IKKα antibody (M-280, Santa Cruz Biotechnology) at 4 °C for 2 h. For IP assay of SGK1 and p300, the clarified hippocampal lysate (1 mg) was immunoprecipitated with 4 µl of anti-phospho-Thr-256-SGK1 antibody (Upstate Biotechnology, Inc.) or anti-p300 antibody (Upstate Biotechnology, Inc.) at 4 °C overnight. The protein G–agarose beads (100 µl, 50% slurry; GE Healthcare) were added to the IP reaction product to catch the immune complexes at 4 °C for 2 h. The immune complexes on beads were then washed three times with washing buffer containing 20 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% IGEPA. CS–630, 1 mM DTT, 50 mM β-glycerophosphate, 50 mM NaF, 10 µg/ml PMSF, 4 µg/ml aprotinin, 4 µg/ml leupeptin, and 4 µg/ml pepstatin.

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of lysate from cell lines or hippocampal tissue was incubated with 10 µl of anti-p65 antibody (C-20, Santa Cruz Biotechnology), 10 µl of anti-FLAG M2-agarose affinity gel (50% slurry) (Sigma), or anti-HA agarose affinity gel (50% slurry) (Sigma) at 4 °C for 2 h, respectively. The immune complexes of p65 were precipitated by using the protein G-agarose beads (50 µl, 50% slurry; GE Healthcare) at 4 °C for 2 h. Antibodies used in this study included Akt-pSub (Cell Signaling), pSGK1 Thr-256, IKKα, IKKβ (Upstate Biotechnology, Inc.), pIKKα/β Thr-23 (Santa Cruz Biotechnology), pIKKα/β Ser-180/181 (Cell Signaling), p65 (C-20, Santa Cruz Biotechnology), acetylated lysine (Cell Signaling), NR1 (Upstate Biotechnology, Inc.), NR2A (Chemicon), NR2B (Chemicon), V5 (Serotec), FLAG M2 (Sigma), and β-actin (Chemicon) antibodies. After washing, blots were incubated with horseradish peroxidase-conjugated secondary antibodies (Chemicon) and were exposed to x-ray film (Eastman Kodak Co.) or LAS-3000 imaging system (Fujifilm, Tokyo, Japan) for visualization of protein bands by enhanced chemiluminescence (ECL PLUS, GE Healthcare). The protein bands were quantified by using the NIH Image J software.

Luciferase Reporter Assay—Luciferase assay was performed in PC12 cells transfected with SGKS2422D, IKKα constructs, IKKβ constructs, p300 constructs, pNF-κB-Luc (Stratagene), pRG-TK (Promega), NR2A promoter construct, NR2B promoter construct, and pGL4.10 (Promega) according to the manufacturer’s protocols (Promega). The relative activity was normalized by Renilla luciferase activity.

GST and His6 Fusion Protein Purification—GST-IκBα-(1–54) Construct
For construction of the GST-p300 (1714–1883) plasmid, the p300 fragment (p300 (1714–1883) containing residue 1829–1835 of the p300 protein) was subcloned into the pGEX-4T-1 vector (pGEX-p300). The mutant p300 plasmid (pGEX-p300S180A) was generated by using the QuickChange site-directed mutagenesis kit (Stratagene). The GST-IκBα-(1–54) plasmid containing residues 1–54 of the IκBα protein was a gift from Dr. Shu Chien. For construction of the His-IKKα, His-IKKαT23A, and His-IKKαS180A plasmids, full-length IKKα was subcloned into the pRSET-A vector (pRSET-IKKα). The mutant IKKα plasmids (pRSET-IKKαT23A and pRSET-IKKαS180A) were generated by using the QuickChange site-directed mutagenesis kit (Stratagene). Bacteria culture (200 ml) of Rosetta-gami 2(DE3)pLysS (Novagen, WI) transformed with the pCG-IKKαWT or FLAG-IKKαT23A plasmid. Twenty four hours after transfection, cell lysates were prepared from bacteria grown in full medium or in full medium treated with 50 µM LYS49002 for 2 h were incubated with FLAG M2-agarose affinity gel (Sigma) at 4 °C for overnight. The immune complexes on beads were then washed three times in wash buffer and twice in reaction buffer containing 20 mM HEPES (pH 7.4), 10 mM MgCl2, and 0.5 mM EGTA. Kinase reaction was carried out in 20 µl of reaction buffer added with 1 mM DTT, 100 µM ATP, and 60 ng of activated SGK1 protein (Upstate Biotechnology, Inc.) for 30 min at 30 °C. Reactions were stopped by boiling in Laemmli buffer followed by Western blot analysis with anti-IKKα antibody (Cell Signaling). To obtain the FLAG-IKKα fusion protein for in vitro kinase assay, HEK293T cells were transfected with FLAG-IKKαWT or FLAG-IKKαT23A plasmid. Fourteen hours after transfection, cell lysates were prepared from bacteria grown in full medium or in full medium treated with 50 µM LYS49002 for 2 h were incubated with FLAG M2-agarose affinity gel (Sigma) at 4 °C for overnight. The immune complexes on beads were then washed three times in wash buffer and twice in reaction buffer containing 20 mM HEPES (pH 7.4), 10 mM MgCl2, and 0.5 mM EGTA. Kinase reaction was carried out in 20 µl of reaction buffer added with 1 mM DTT, 100 µM ATP, and 60 ng of activated SGK1 protein (Upstate Biotechnology, Inc.) for 30 min at 30 °C. For the coupling kinase assay, the FLAG-IKKα IP product prepared from HEK293T cells was incubated with 60 ng of activated SGK1 protein (Upstate Biotechnology, Inc.) and 6 µCi of [γ-32P]ATP (3000 Ci/mmol) for 30 min at 30 °C. Reactions were stopped by boiling in Laemmli buffer and subjected to 8% SDS-PAGE followed by transfering onto the PVDF membrane. The membrane was exposed to x-ray film (Kodak) for visualization of protein bands.

Gene Transfection and Drug Injection to the Brain—Adult male Sprague-Dawley rats (250–400 g) were obtained from the Institute of Biomedical Sciences, Academia Sinica, were used. Experimental procedures followed the Guidelines of Animal Use and Care of the National Institute of Health and were approved by the Animal Committee of the Institute of Biomedical Sciences, Academia Sinica. Animals were anesthetized with pentobarbital (40 mg/kg, intraperitoneally) and subjected to stereotactic surgery. Two 23-gauge, stainless steel, thin wall cannulae were implanted bilaterally to the hippocampal CA1 area at the following coordinates: 3.5 mm posterior to the bregma, 2.5 mm lateral to the midline, and 3.4 mm ventral to the skull surface. After animals recovered from the surgery, the pcDNA3 vector or the constitutively active SGK, SGKS2422D, was injected to the
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CA1 area. Before injection, plasmid DNA was diluted in 5% glucose to a stock concentration of 2.77 μg/μl. Branched polyethyleneimine of 25 kDa (Sigma) was used. The volume for DNA injection was 0.5 μl each side at a concentration of 1.2 μg/μl. The volume for IGF-1 injection was also 0.5 μl each side at a concentration of 100 ng/ml. Rat SGK1 siRNA was designed according to a previous report (18). The siRNA sequences of rat SGK1 was synthesized by Ambion with the following sequences: sgk-1 sense oligonucleotide, 5'-GUCGCUCCAACAAUAACATC-3'; antisense oligonucleotide, 5'-UGGAUUUGUAGGAGGAG-3' (8). The siRNA sequences for rat Akt was synthesized by MBDIO (Taipei, Taiwan) with the following sequences: sense oligonucleotide, 5'-UGCCCUUCUACAACCAGGGATT-3'; antisense oligonucleotide, 5'-UCCUGGUUGUAGGAGGCATT-3' (19). Silencer Negative Control number 1 siRNA (Ambion) was used as a control. SGK1 siRNA and negative control siRNA (8 pmol/μl each) were transfected into the hippocampus (0.5 μl each side) by using the cationic polymer transfection reagent jetSTM 10 mM (Polyplus-Transfection). The injection rate was at 0.2 μl/min. Animals were sacrificed 48 h after DNA transfection or 30 min after IGF-1 injection. For combined treatment, SGK1 siRNA or Akt siRNA was given 96 h before IGF-1 treatment. The hippocampal CA1 tissue was dissected for Western blot analyses of IKKβ (20). The primers and TaqMan probe of HPRT were synthesized by Applied Biosystems with the following sequences: forward primer 5'-GCCGATCTTACAGGCTTAAAC-3' and reverse primer 5'-TGACCAGAATTTGGTGAGG-3'. The primers of NR2B were synthesized with the following sequence: forward primer 5'-CAAGAAGATCGCCCAACCT-3' and reverse primer 5'-GTTACAGACATTGGTCTGCTG-3'. Amplification was performed by using the 7500 Real Time PCR system (Applied Biosystems), and the reaction condition followed the manufacturer's protocols. The thermal cycler protocol used is as follows: stage 1, 50 °C for 2 min; stage 2, 95 °C for 10 min; stage 3, 95 °C for 15 s, 60 °C for 1 min 40 cycles); and stage 4 is the dissociation stage for SYBR Green fluorescence signal, and the result was collected by reading the absorbance at 450 nm.

FIGURE 1. Putative substrate proteins of SGK1. Tissue lysate (10 μg) from rat dorsal hippocampus was treated with activated SGK1 protein (0, 20, 40, or 100 ng) and ATP (100 μm) for 10 min and subjected to kinase reaction and Western blot (WB) by using the p-motif (p-RRXXS/T) antibody. Protein bands that are recognized by this antibody, and the densities that are increased by activated SGK1 are indicated by the asterisk. Actin was used as an internal control. Experiments are in triplicate.

No Shift Transcription Factor Assay—A rapid and sensitive way to measure NF-κB activity in hippocampal nuclear extract without isotopes or gels was also carried out by using the No-shift transcription factor assay kit with NF-κB reagent (Novagen). In this assay, the biotinylated oligonucleotides with NF-κB consensus sequence and NF-κB in the nuclear extracts form complex first. The NF-κB (p65)-specific antibody was then used to detect the NF-κB-DNA complex captured on the streptavidin plate. Finally, the appropriate secondary antibody, HRP conjugate, and TMB substrate were used to develop colorimetric signal, and the result was collected by reading the absorbance at 450 nm.
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Nuclear Extract Preparation—Briefly, 90–120 mg of hippocampal tissue was homogenized with a Dounce tissue homogenizer (Wheaton, NJ) in a homogenization buffer (10% w/v) containing 20 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 0.5 mM EDTA, 1% IGEPAL CA-630, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 5 µg/ml aprotinin, and 2.5 µg/ml leupeptin. The homogenate was centrifuged at 1,000 g for 5 min at 4 °C. The resulting supernatant, which contains DNA-binding proteins, was carefully removed and stored at −80 °C until further use.

Spatial Learning—The Morris water maze learning paradigm was used in this study. The water maze was a plastic circular pool (2.0 m diameter and 0.6 m height) filled with water (25 ± 2 °C) to a depth of 20 cm. A circular platform (13.5 cm diameter) was placed at a specific location away from the edge of the pool. The top of the platform was submerged 1.5 cm below the water surface. Water was made cloudy by the addition of milk powder. Distinctive visual cues were set on the wall.

For spatial learning, animals were subjected to three trials a day with one given early in the morning, one given in the early afternoon, and another given in the late afternoon. The training procedure lasted for 4 days, and a total of 12 trials was given. For these trials, animals were placed at different starting positions spaced equally around the perimeter of the pool in a random order. Animals were given 60 s to find the platform. If an animal could not find the platform, it was guided to the platform. After mounting the platform, animals were allowed to stay there for 20 s. The time that each animal took to reach the platform was recorded as the escape latency. A probe trial of 60 s was given on day 5 to test their memory retention.

Animals were placed in the pool with the platform removed, and the time the animals spent in each quadrant (target quadrant, left quadrant, opposite quadrant, and right quadrant) was recorded.
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A. In vitro kinase assay

- + - + Δ SGK
- + + + Flag-IKKαWT
- + + + GST-IκBα(1-54)

70 kDa

pIKKα (autoradiography)

35 kDa

pIKBα (autoradiography)

Flag (western blot)

GST (western blot)

B. HEK293T cell

+ + + + Flag-p65
- + + + Flag-IKKαWT
- + + + Flag-IKKαT23A
- + + + Flag-IKKαS180A
- + + + Flag-IKKαT23AS180A
- + + + Flag-IKKαK44M

HA-SGKS422D

- 0.8 0.8 0.8 0.8

p65 S536

p65 S529

plκB (anti-Flag)

IκBα

p50 (anti-Flag)

HA

Actin

C. HEK293T cell

+ + 0.4 0.8 0.8 0.8 0.8

Flag-p65

HA-SGKS422D

V5-IKKβWT

V5-IKKβK44M

p65 S536

Flag

plκB

IκBα

V5

HA

D. Reporter assay

Relative activity (fold)

pcDNA3

HA-SGK S422D

HA-SGK S422D

HA-SGK S422D

HA-SGK S422D

pCMV Tag2A

pCMV Tag2A

Flag-IKKαWT

Flag-IKKαT23A

Flag-IKKαS180A

Flag-IKKαT23AS180A

Flag-IKKαK44M

HA

Flag

E. Reporter assay

Relative activity (fold)

PC3 DNA3

Flag

pCMV Tag2A

Flag-IKKαWT

Flag-IKKαT23A

Flag-IKKαS180A

Flag-IKKαT23AS180A

Flag-IKKαK44M

Flag

F. HEK293T cell

- + + Insulin

SGK siRNA

IGF-1

Akt siRNA

- + + Insulin

Akt siRNA

pIKKαT23

IKKα

p65 S536

p65

SGK

Actin

G. Reporter assay

Relative activity (fold)

Control

IGF-1

SGK siRNA

Akt siRNA

- + + Insulin

Neg siRNA

Neg siRNA

Actin

Actin
sequence RXRX(S/T), where X stands for any amino acid, we have used the p-motif antibody that recognizes phosphorylated proteins containing the RXRX(S/T) motif to identify the substrates phosphorylated by SGK1. Cell lysates from rat hippocampus were added with different amounts of activated SGK1 protein and subjected to kinase reaction and immunoblot analysis. By doing this, several protein bands were identified as shown in Fig. 1. We have further used the protein data base motif search engine Scansite (22) to predict these proteins according to the approximate molecular weight of each band shown in Fig. 1. Based on the result of substrate prediction, the entire predicted candidate proteins were divided into three molecular weight ranges as shown in supplemental Table 1. Among these proteins, GSK-3β and FOXO3A are possibly two of the known candidate proteins of SGK1 (3, 23), but inclusion of other proteins also at these molecular weights cannot be excluded. Furthermore, other candidate proteins are not identified yet.

SGK1 Directly Phosphorylates IKKα at Thr-23 and Enhances the Phosphorylation of IKKα at Ser-180—Based on these predictions, we have chosen IKKα (molecular weight around 85 kDa, marked with double asterisks in Fig. 1) as the candidate protein for the present study. To confirm the accuracy of substrate prediction and to examine whether IKKα phosphorylation at Thr-23 was increased in the kinase reaction, we have added different amounts of activated SGK1 protein to hippocampal lysate and examined the level of pIKKαThr-23 by using immunoblot analysis. Because IKKα is a known substrate of Akt (24), activated Akt protein was used as a positive control. Results revealed that the level of pIKKα/β Thr-23 was increased in a dose-dependent manner (supplemental Fig. S1A). Further experiment with FLAG-IKKαWT and V5-IKKβWT transfection to HEK293T cells and Western blot showed that pIKKα/β Thr-23 antibody recognizes IKKα but not IKKβ (Fig. 2A, left). To further distinguish whether the pIKKα/β Thr-23 antibody recognizes the phosphorylated IKKα only, recombinant His-tagged IKKα protein was incubated with or without activated SGK1 for in vitro kinase assay. Results revealed that this antibody only recognizes the phosphorylated IKKα but not the nonphosphorylated IKKα (Fig. 2A, right). These results suggest that IKKα may be a downstream target of SGK1.

Next, we examined whether IKKα is a direct target of SGK1. Anti-IKKα and anti-Thr(−P)-256 SGK1 antibodies were used to immunoprecipitate (IP) endogenous IKKα and SGK1 from hippocampal lysate. Results from co-IP experiment revealed that SGK1 forms a complex with IKKα (Fig. 2B). Immunoblotting for SGK1 under pSGK immunoprecipitation was not carried out because it is not distinguishable whether the band is SGK1 or the heavy chain of immunoglobulin. We then examined whether SGK1 phosphorylates IKKα in vitro and in vivo. When activated SGK1 was incubated with His-tagged wild-type (WT) and T23A mutant recombinant IKKα proteins, only IKKαWT was phosphorylated by SGK1 but not the T23A mutant (Fig. 2C). Furthermore, when activated SGK1 was incubated with GST-tagged IKKβ, no phosphorylation signal was detected (supplemental Fig. S1B). Western blot showed that Ser-181 of IKKβ was not phosphorylated by SGK1 either (supplemental Fig. S1B). The dot seen on the gel is still a nonspecific band even when visualized at a higher intensity (supplemental Fig. S2). These results indicated that SGK1 only phosphorylates IKKα but not IKKβ. Results from IP kinase assay also revealed that SGK1 phosphorylates IKKα at Thr-23 (supplemental Fig. S1C). Further experiments in HEK293T cells revealed that phosphorylation of IKKα at Thr-23 and Ser-180 was both increased after co-transfection of the constitutively active SGK1, SGKS422D, and IKKα (Fig. 2D). Transfection of the IKKαT23A mutant seemed to slightly diminish the effect of SGKS422D on Ser-180 phosphorylation, but SGKS422D at the highest concentration (1.2 μg) still apparently increased Ser-180 phosphorylation under IKKαT23A (Fig. 2D). Although SGK1 enhances the phosphorylation of IKKα at Ser-180, further in vitro kinase assay revealed that SGK1 does not directly phosphorylate Ser-180 of IKKα (supplemental Fig. S1D). In another study, it is shown that SGK1 phosphorylates IKKβ at Ser-181 (25). Because Ser-181 does not fit into the RXRX(S/T) motif, we have re-examined this issue by co-transfection of IKKβ and SGKS422D to HEK293T cells. Results revealed that IKKβ Ser-181 is not phosphorylated by SGK1 in vivo (supplemental Fig. S1E). The beads seen with IKKαWT transfection alone in Fig. 2D and with IKKβWT transfection alone in supplemental Fig. S1E are probably because of endogenous phosphorylation of IKKα and IKKβ in HEK293 cells by endogenous kinases.

The above results demonstrated that SGK1 phosphorylates IKKα in vitro and in vivo, but they do not reveal whether this event also occurs under physiological conditions. This issue was examined here. IGF-1 was shown to induce SGK1 activation (11) and NF-κB activity (26) and was used here as an upstream signal of SGK1. Results from Western blot showed that injection of IGF-1 (100 ng/ml) to hippocampal CA1 area

![FIGURE 3. SGK1 enhances NF-κB activity through IKKα and IKKβ](image-url)
significantly increased IKKα phosphorylation at Thr-23 ($F_{2,9} = 26.44, p < 0.01, q = 6.91, p < 0.01$) without affecting the IKKα protein level (Fig. 2E). To verify that IGF-1-induced phosphorylation of IKKα is mediated through SGK1, SGK1 siRNA (4 pmol) was administered to hippocampal CA1 area 96 h before IGF-1 injection. Results revealed that SGK1 siRNA pretreatment blocked the effect of IGF-1 on IKKα phosphorylation at Thr-23 ($q = 5.26, p < 0.01$ when comparing IGF-1+SGK1
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siRNA group with IGF-1 group) without affecting the IKKα protein level (Fig. 2E). The effectiveness of SGK1 siRNA treatment was confirmed by an apparent decrease of SGK1 protein level in the hippocampus (Fig. 2E).

SGK1 Up-regulates NF-κB Activity through Phosphorylation of IKKα—Next, we examined whether SGK1 phosphorylation of IKKα regulates NF-κB activity. In the coupling kinase assay carried out in HEK293T cells, phosphorylation of Ikβα was increased when IKKα was first phosphorylated by SGK1 and then incubated with recombinant Ikβα (Fig. 3A). This result suggests that SGK1 phosphorylates IKKα and consequently up-regulates IKK complex activity. A light band of pIKKα and pIkβ without activated SGK1 is also seen here (Fig. 3A, 3rd lane). This is probably because of phosphorylation of IKKα by endogenous kinases in HEK293T cells, such as SGK1 and Akt. It could also be due to autophosphorylation of these proteins. Because IKKα is responsible for the phosphorylation of p65 at Ser-529 and Ser-536, and this phosphorylation is important for the transcriptional activity of NF-κB (27), we then examined whether phosphorylation of p65 at Ser-529 and Ser-536 is regulated by SGK1. We also examined whether Ikβ phosphorylation is regulated by SGK1 through IKKα. Results from Western blot revealed that SGK1 increased the phosphorylation of p65 at both Ser-529 and Ser-536, and this effect is dependent on the activity of IKKα (Fig. 3B). In addition, phosphorylation of IKKα at Thr-23 and Ser-180 seems equally important in SGK1-mediated NF-κB phosphorylation at Ser-529, but IKKα phosphorylation at Ser-180 seems to play a major role in SGK1-mediated NF-κB phosphorylation at Ser-536 (Fig. 3B). SGK1 also increased the phosphorylation of Ikβ that is further potentiated by co-transfection of IKKα. Transfection of IKKαT23A and IKKαS180A both diminished the effect of Ikβ phosphorylation induced by SGK1. In particular, IKKαS180A had a more significant effect. But co-transfection of the IKKα double mutant (IKKαT23A/S180A) and IKKα kinase-dead mutant (IKKαK44M) both completely blocked this effect of SGK1. The Ikβ protein level was not affected by these manipulations (Fig. 3B). Further transfection experiment showed that IKKβ is also required for the effect of SGK1 on p65 Ser-536 phosphorylation (Fig. 3C). These results together suggest that although SGK1 does not phosphorylate IKKβ, the kinase activity of IKKβ still has to be present for IKK complex activation. IKKα or IKKβ alone is not sufficient for IKK complex activation. In the NF-κB reporter assay, transfection of SGKS422D to PC12 cells markedly increased NF-κB activity (one-way ANOVA and Dunnett’s t test, \( t = 3.25, p < 0.01 \)) and this effect was further enhanced by IKKαWT transfection (\( q = 5.69, p < 0.01 \), Newman-Keul’s statistics) (Fig. 3D, upper). Transfection of IKKαT23A alone and IKKαS180A alone both partially blocked the effect of SGKS422D on NF-κB activity, but transfection of IKKαT23A/S180A and IKKαK44M both abolished the effect of SGKS422D on NF-κB activity (\( q = 3.12, p < 0.05 \) and \( q = 5.55, p < 0.01 \)) (Fig. 3D, upper). Western blot for anti-HA and anti-FLAG was used for verification of plasmid transfection and expression (Fig. 3D, lower). To further confirm the effect of SGK1 on NF-κB activation, we have performed an electrophoretic mobility shift assay experiment, and the result consistently showed that SGKS422D increased NF-κB activity (supplemental Fig. S3). But for the purpose of quantification, we have used the luciferase reporter assay for the following experiments. This result suggests that IKKα phosphorylation at Thr-23 and Ser-180 plays a role in NF-κB activation. Similar results were observed when SGKS422D, IKKβWT, IKKβS181A mutant, and IKKβ kinase-dead mutant were transfected to PC12 cells (Fig. 3E). Transfection of SGKS422D to PC12 cells markedly increased NF-κB activity (one-way ANOVA and Dunnett’s \( t \) test, \( tD = 4.44, p < 0.05 \)), and this effect was further enhanced by IKKβWT transfection (\( q = 8.93, p < 0.001 \)) (Fig. 3E, upper). Although transfection of IKKβS181A only partially blocked the effect of SGKS422D + IKKβWT on NF-κB activity, IKKβ kinase-dead mutant (IKKβK44M) completely abolished the effect of SGKS422D on NF-κB activity (\( q = 8.543, p < 0.001 \) and \( q = 10.482, p < 0.001 \)) (Fig. 3E, upper). Similarly, Western blot for anti-HA and anti-V5 was used for verification of plasmid transfection and expression (Fig. 3E, lower). This latter result supported the notion that IKKβ kinase activity is also required for SGK1 activation of NF-κB, and these results together suggest that phosphorylation of IKKα at Thr-23/Ser-180, IKKα, and IKKβ activity are all required for SGK1-mediated NF-κB activation.

Next, we examined whether SGK1 is involved in NF-κB activation under physiological conditions. Insulin was shown to activate both SGK1 (11) and NF-κB (28) and was used here to examine this issue. Because protein kinase Akt was shown to share 50% homology to the catalytic domain of SGK1 (23), and it also up-regulates NF-κB activity (29), Akt was used as a positive control here. Results from Western blot indicated that insulin treatment (100 ng/ml) to HEK293T cells increased IKKα phosphorylation at Thr-23 and NF-κB phosphorylation at Ser-536 without affecting their protein levels (Fig. 3F). But SGK1 siRNA pretreatment (20 pmol) and Akt siRNA pretreatment (20 pmol) both blocked these effects of insulin (Fig. 3F).

FIGURE 4. SGK1 promotes NF-κB acetylation through phosphorylation of p300. A, HEK293T cells were transfected with pcDNA3 or SGKS422D (1.6 μg) 48 h before cell extraction, and cell extracts (500 μg) were prepared for p65 IP assays by using the acetylated lysine antibody, B, HA-SGK (1.2 μg) and FLAG-p300 (0.4 μg) was co-transfected to HEK293T cells 48 h before cell extraction, and the cell extracts (500 μg) were subjected to co-IP assay and Western blot against FLAG-p300 and HA-SGK. C, protein extract from hippocampal tissue (1 mg) for co-IP assay was prepared from animals receiving IGF-1 injection (100 ng/ml) to the hippocampus (right). D, GST-tagged p300 and GST-tagged p300S1834A fusion proteins were incubated with activated SGK1 (60 ng) and 6 μCi of \( [\gamma-\text{32P}]\text{ATP} \) for 30 min for kinase reaction and Western blot by using the GST antibody. E, FLAG-p300WT (0.4 μg) or FLAG-p300S1834A (0.4 μg) alone or in combination with HA-SGKS422D (1.2 μg) was transfected to PC12 cells 48 h before NF-κB reporter assay. F, different combination of plasmids FLAG-p65 (0.4 μg) alone or in combination with HA-SGKS422D (0.8 μg) and FLAG-p300WT (0.4 μg) or FLAG-p300S1834A (0.4 μg) was transfected to HEK293T cells 48 h before protein extraction for IP-Western against FLAG and acetylated p65. G, SGK1 siRNA (50 pmol and 150 pmol) was transfected to HEK293T cells (on a 6-well plate) 48 h before insulin treatment (100 ng/ml), and NF-κB acetylation assay was performed 30 min after insulin treatment. H, various combinations of IKKα, IKKαT23A, SGKS422D, p300, and p300S1834A plasmid was transfected to HEK293T cells to examine whether SGK1 phosphorylation of IKKα Thr-23 is dependent on a prior SGK1 phosphorylation of p300 (upper) and vice versa (lower). Experiments are in duplicate or triplicate. Data and statistical significance are expressed as in Fig. 3. Data are means ± S.E. #, \( p < 0.01 \) compared with the control group; *, \( p < 0.05 \); **, \( p < 0.01 \); and ***, \( p < 0.001 \).
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The effectiveness of SGK1 siRNA and Akt siRNA treatments was confirmed by an apparent reduction of SGK1 and Akt protein level in HEK293T cells, respectively (Fig. 3F).

We next examined whether SGK1 mediates the effect of IGF-1 on NF-κB activation. Akt was also used as a positive control. Results revealed that IGF-1 treatment (100 ng/ml) significantly increased NF-κB promoter activity in PC12 cells (F = 13.2, p < 0.01, q = 9.41, p < 0.01). But transfection of SGK1 siRNA (20 pmol) and Akt siRNA (20 pmol) both completely antagonized the effect IGF-1 on NF-κB promoter activity (q = 8.75, p < 0.01, and q = 13.9, p < 0.01, respectively) (Fig. 3G). These results together with results from Fig. 3F suggest that although both SGK1 and Akt mediate IKKα phosphorylation and NF-κB activation; however, blockade of either SGK1 signaling or Akt signaling would prevent NF-κB activation resulted from upstream stimulation.

SGK1 Promotes NF-κB Acetylation through Phosphorylation of p300—Because phosphorylation and acetylation of NF-κB are both important for NF-κB DNA binding activity (30), and p300 activation enhances NF-κB p65 acetylation (27), in this experiment we examined whether SGK1 enhances NF-κB activity through the mediation of p300. We first found that SGK422D transfection to HEK293T cells increased the acetylation level of NF-κB (Fig. 4A). Because p300 contains one RXRXX(S/T) motif (1829RrRmaSm1835) that could be phosphorylated by SGK1, we then examined whether SGK1 may enhance NF-κB acetylation through phosphorylation of p300. Co-IP experiments of overexpression of HA-SGK1 and FLAG-p300 revealed that p300 forms a complex with SGK1 in HEK293T cells (Fig. 4B). Further co-IP experiments from hippocampal tissue lysate showed that SGK1 is also associated with p300 in the hippocampus (Fig. 4C, left), and the association between SGK1 and p300 in the hippocampus is increased upon IGF-1 injection to hippocampal neurons (Fig. 4C, right). This result suggests that the association between SGK1 and p300 is up-regulated by IGF-1 physiologically. Moreover, results from in vitro kinase assay revealed that p300 is phosphorylated directly by SGK1 at Ser-1834 (Fig. 4D). Furthermore, SGK422D and p300 both increased NF-κB activity, and SGK422D and p300 also cooperated to up-regulate NF-κB activity in the reporter assay in PC12 cells (p < 0.01) (Fig. 4E). But transfection of the p300 mutant (p300S1834A) antagonized the effect of SGK422D on NF-κB activity (q = 4.1, p < 0.05) (Fig. 4E). This latter result suggests that SGK1 may also regulate the acetyltransferase activity of p300. To test this hypothesis, we have transfected SGK422D and p300 to HEK293T cells and examined the acetylation level of p65. Results revealed that p65 acetylation was increased upon transfection of p300 (q = 7.01, p < 0.01). This effect was further potentiated by SGK422D co-transfection (q = 15.39, p < 0.001) but was antagonized by co-transfection of the p300 mutant (p300S1834A) with or without SGK422D co-transfection (q = 0.11 and 1.4, both p > 0.05) (Fig. 4F). IGF-1 was found to increase NF-κB promoter activity (Fig. 3G), and here we examined whether insulin also increases NF-κB acetylation and whether this is mediated through SGK1. Results revealed that insulin (100 ng/ml) treatment to HEK293T cells apparently increased p65 acetylation, but this effect was blocked by SGK1 siRNA pretreatment in a dose-dependent manner (Fig. 4G). SGK1 siRNA alone at a higher concentration (150 pmol) also decreased p65 acetylation (Fig. 4G). The p65 protein level was not altered by these treatments. On the other hand, insulin did not apparently increase p300 phosphorylation at Ser-1834, but SGK1 siRNA treatment decreased p300 phosphorylation (Fig. 4G). This latter result suggests that a mechanism other than SGK1 is involved in the action of insulin on p300 phosphorylation. Finally, we examined whether SGK1 phosphorylation of IKKα Thr-23 depends on a prior phosphorylation of p300 by SGK1 and vice versa. Results from a co-transfection experiment in HEK293T cells revealed that SGK422D consistently increased IKKα phosphorylation at Thr-23, but co-transfection of p300S1834A did not alter this effect of SGK422D (Fig. 4H, upper). Likewise, SGK422D increased the phosphorylation of p300 at Ser-1834, but transfection of IKKαT23A did not alter this effect of SGK422D (Fig. 4H, lower, 2nd and 4th lanes). Together with a previous result that SGK1 activation of IKKα at Ser-180 is independent of SGK1 phosphorylation of IKKα at Thr-23 (Fig. 2D), these results suggest that SGK1 activation of IKKα at Thr-23, Ser-180, and SGK1 activation of p300 are independent of each other.

SGK1 Up-regulates NMDA Receptor NR2A and NR2B Expression through IKKα, p300, and NF-κB Mediation—After identification of SGK1-IKKα-NF-κB signaling and SGK1-p300-NF-κB signaling, we next examined gene expressions that are regulated by these signaling pathways. Because SGK1 (10), NF-κB (31), and the NMDA receptors (32) all play an important role in learning and memory function, and the promoters of NMDA receptor NR1 and NR2A may contain the NF-κB binding elements based on an earlier study of the NR1 promoter (33) and the transcription element search system (Fig. 5A), we therefore examined whether these signaling pathways may regulate NMDA receptor subunit expression. SGK422D was first transfected to rat hippocampal neurons, and the expression of different NMDA receptor subtypes was examined by real time PCR. Results revealed that SGK422D transfection markedly increased the expression of NR1, NR2A, and NR2B (t1,8 = 4.839, 4.855, and 4.753, respectively; all p < 0.001) (Fig. 5B, left). Immunoprecipitation of HA followed by immunoblotting with anti-SGK1 antibody confirmed the expression of SGK422D in hippocampal neurons (Fig. 5B, right). Different cell lines were then used for the following experiments for gene expression analyses because NR1, NR2A, and NR2B are differentially expressed in different cells. Results obtained in PC12 cells and Neuro2A cells revealed that SGK422D increased the expression of NR1 (Fig. 5C), NR2A, and NR2B (Fig. 5D). But this effect is blocked by transfection of the kinase-dead SGK1, SGK1K127M (p > 0.05 compared with controls) (Fig. 5, C and D). To further examine the role of SGK1 on NR1, NR2A, and NR2B expression, we have transfected SGK1 siRNA (20 pmol) to PC12 cells (for NR1) and Neuro2A cells (for NR2A and NR2B) and examined NR1, NR2A, and NR2B mRNA level by using real time PCR. Results showed that SGK1 siRNA significantly decreased the mRNA level of NR1, NR2A, and NR2B (t1,8 = 4.23, 3.99, and 5.56, respectively; all p < 0.01) (Fig. 5E). We further examined whether SGK1 siRNA affects NR1, NR2A, and NR2B protein level in the hippocampus. SGK1
siRNA (4 pmol) was transfected to hippocampal CA1 area, and Western blot was carried out. Results showed that SGK1 siRNA apparently decreased NR1, NR2A, and NR2B protein level in the hippocampus (Fig. 5F). The effectiveness of SGK1 siRNA transfection was confirmed by decreased SGK1 protein level. We also examined the effect of Akt siRNA on NR1, NR2A, and NR2B mRNA expression. Results showed that Akt siRNA (20 pmol) had a marginal effect on NR1 mRNA expression ($t_{1,10} = 1.84$ and 1.14, both $p > 0.05$), and it only significantly decreased NR2B mRNA expression ($t_{1,10} = 3.53, p < 0.01$) (Fig. 5G). Akt siRNA at a higher concentration (50 pmol) did not further decrease NR2A and NR2B mRNA levels (supplemental Fig. S4). We then examined whether IGF-1 may up-regulate NR1, NR2A, and NR2B mRNA expression, and whether this effect is mediated through SGK1. Results revealed that IGF-1 (100 ng/ml) administration to PC12 cells and Neuro2A cells did not alter NR1, NR2A, and NR2B mRNA expression at all ($p > 0.05$; Fig. 5H). Therefore, we did not perform a further SGK1 siRNA and IGF-1 interaction study.

Next, we examined whether SGK1 regulates NR1, NR2A, and NR2B expression through NF-κB. SGKS422D was transfected to PC12 cells and Neuro2A cells with or without SN50, an NF-κB inhibitor. Results revealed that SN50 did not alter the effect of SGKS422D on NR1 (Fig. 6A), but it blocked the effect of SGKS422D on NR2A and NR2B expression (Fig. 6B). The lack of an effect of SN50 in blocking SGKS422D effect on NR1 expression could be due to the possibility that the NF-κB-binding site on NR1 promoter is not functionally activated. We next examined whether NR2A and NR2B expression is also regulated by SGK1 phosphorylation of IKKα and p300. Various combinations of SGKS422D and IKKα mutant plasmids were transfected to Neuro2A cells, and the expression of NR2A and NR2B was exam-
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SGK1 up-regulates NMDA receptor NR2A and NR2B expression through IKKα, NF-κB, and p300 mediation. A, SGK422D (1.6 μg) was transfected to PC12 cells 48 h before RNA extraction, and SN50 (an NF-κB inhibitor, 50 μg/ml; Calbiochem), alone or in combination with HA-SGK422D, was added to the same PC12 cells 1 h before RNA extraction. Real time PCR was performed to determine NR1 mRNA levels. B, SGK422D (1.6 μg) was transfected to Neuro2A cells 48 h before RNA extraction, and SN50 (50 μg/ml), alone or in combination with SGK422D, was added to the same cells 1 h before RNA extraction. Real time PCR was performed to determine NR1 mRNA levels. C, SGK422D (1.2 μg), IKKα WT (0.4 μg), IKKαT23A (0.4 μg), IKKαS180A (0.4 μg), IKKα double mutant (0.4 μg), and combined SGK422D + various IKKα mutant plasmids were transfected to Neuro2A cells 48 h before RNA extraction. Real time PCR was performed to determine NR2A and NR2B mRNA expressions. D, SGK422D (1.2 μg), p300 WT (0.4 μg), p300 S1834A (0.4 μg), and the combined treatment of SGK422D + p300 S1834A were transfected to Neuro2A cells 48 h before RNA extraction. Real time PCR was performed to determine NR2A and NR2B mRNA levels. E, SGK422D (1.2 μg), p65 (0.4 μg), the p65 acetylation mutant p65K221R (0.4 μg), and combined SGK422D + p65K221R plasmid were transfected to Neuro2A cells 48 h before RNA extraction. Real time PCR was performed to determine NR2A and NR2B mRNA levels. Experiments are in triplicate. Data and statistical significance are expressed as in Fig. 3. Data are means ± S.E. #, p < 0.05; ##, p < 0.01 compared with the control group. ***, p < 0.001.

FIGURE 6. SGK1 up-regulates NMDA receptor NR2A and NR2B expression through IKKα, NF-κB, and p300 mediation. A, SGK422D (1.6 μg) was transfected to PC12 cells 48 h before RNA extraction, and SN50 (an NF-κB inhibitor, 50 μg/ml; Calbiochem), alone or in combination with HA-SGK422D, was added to the same PC12 cells 1 h before RNA extraction. Real time PCR was performed to determine NR1 mRNA level. B, SGK422D (1.6 μg) was transfected to Neuro2A cells 1 h before RNA extraction. Real time PCR was performed to determine NR2A and NR2B mRNA levels. C, SGK422D (1.2 μg), IKKα WT (0.4 μg), IKKαT23A (0.4 μg), IKKαS180A (0.4 μg), IKKα double mutant (0.4 μg), and combined SGK422D + various IKKα mutant plasmids were transfected to Neuro2A cells 48 h before RNA extraction. Real time PCR was performed to determine NR2A and NR2B mRNA expressions. D, SGK422D (1.2 μg), p300 WT (0.4 μg), p300 S1834A (0.4 μg), and the combined treatment of SGK422D + p300 S1834A were transfected to Neuro2A cells 48 h before RNA extraction. Real time PCR was performed to determine NR2A and NR2B mRNA levels. E, SGK422D (1.2 μg), p65 (0.4 μg), the p65 acetylation mutant p65K221R (0.4 μg), and combined SGK422D + p65K221R plasmid were transfected to Neuro2A cells 48 h before RNA extraction. Real time PCR was performed to determine NR2A and NR2B mRNA levels. Experiments are in triplicate. Data and statistical significance are expressed as in Fig. 3. Data are means ± S.E. #, p < 0.05; ##, p < 0.01 compared with the control group. ***, p < 0.001.
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FIGURE 7. SGK1 increases NR2A and NR2B promoter activity. A, NR2A promoter construct (0.8 μg) in the length of 0.5K with or without (Δ) the NF-κB-binding site was transfected to Neuro2A cells, and NR2A promoter activity was determined by luciferase reporter assay 48 h after transfection. B, SGKS422D (0.8 μg) was co-transfected with the NR2A promoter construct with or without the NF-κB-binding site (0.6 μg) to Neuro2A cells, and NR2A promoter activity was determined 48 h later by luciferase reporter assay. C, different lengths of the NR2B promoter construct (0.5K, 1.0K, 1K, 1.5K, 2K, 2.5K, and 3K; where K means thousand base pairs of promoter length) (0.8 μg each) was transfected to Neuro2A cells, and NR2B promoter activity was determined by luciferase reporter assay 48 h later. D, SGKS422D (0.8 μg) or SGK1 siRNA (20 pmol) was co-transfected with the 1.5K length NR2B promoter construct to Neuro2A cells, and NR2B promoter activity was determined 48 h later by luciferase reporter assay. Data and statistical significance are expressed as in Fig. 3. Data are means ± S.E. ##, p < 0.01; ###, p < 0.001 compared with the control group.

-3000; 1000 bp, nt −2020 to −3000; 500 bp, nt −2510 to −3000). Similarly, we have cloned the rat NR2A promoter construct (0.5K, nt +1 to −500, see Fig. 5A) and the rat NR2A promoter construct with deleted NF-κB-binding sites (Δ) to examine whether NR2A promoter activity is regulated by SGK1 through NF-κB. These promoter constructs were then transfected to Neuro2A cells alone or in combination with SGKS422D. The results revealed that transfection of the NR2A promoter construct alone increased NR2A promoter activity about 2.8-fold, but this effect was reversed when the NR2A promoter construct containing deleted NF-κB-binding sites was transfected (Fig. 7A). Furthermore, co-transfection of SGKS422D and NR2A promoter constructs markedly increased NR2A promoter activity ($q = 6.01, p < 0.001$), but this effect was reversed by co-transfection of the NR2A promoter construct with deleted NF-κB-binding site NR2A promoter ($p > 0.05$ compared with control) (Fig. 7B). We next examined NR2B promoter activity. Different lengths of the NR2B promoter constructs were transfected to Neuro2A cells. Results revealed that when the 1500-bp-long promoter construct was transfected, there was a 5.5-fold increase in NR2B promoter activity (Fig. 7C). Other promoter constructs are without any effect (Fig. 7C). These results suggest that the NR2B promoter sequence between nt −1480 and −2020 contains NF-κB-binding site(s). To further examine whether NR2B promoter activity is regulated by SGK1, we have co-transfected the 1500-bp-long NR2B promoter construct with SGKS422D or SGK1 siRNA. Results revealed that SGKS422D transfection significantly increased NR2B promoter activity for about 6-fold ($p < 0.001$), but SGK1 siRNA transfection markedly decreased NR2B promoter activity ($p < 0.01$) (Fig. 7D).

SGK1 Facilitates Spatial Memory Formation through Phosphorylation of IKKa—Upon identification of the SGK1-IKKα-NF-κB pathway, we then assessed the functional significance of this pathway. Because SGK1 plays an important role in spatial learning (10) and p65 knockout mice show impaired radial arm maze performance (31), we have used the water maze learning task to examine the possible involvement of this pathway in spatial memory formation. We first found that water maze training significantly increased IKKα phosphorylation at Thr-23 and Ser-180 in rat hippocampus ($t = 4.85$ and 3.93, both $p < 0.05$; Student’s $t$ test) (Fig. 8, A and B). Water maze training also significantly increased NF-κB activity in the hippocampus ($t_{1,8} = 3.5, p < 0.05$) (Fig. 8C). We then examined whether IKKα phosphorylation mediates the effect of SGK1 on spatial memory formation. Results revealed that SGKS422D transfection to hippocampal CA1 area markedly enhanced acquisition performance ($F_{3,31} = 3.12, p < 0.05$; $q = 3.71, p < 0.05$). Transfection of the IKKα double mutant alone did not have a significant effect on spatial learning ($q = 0.89, p > 0.05$), but it completely antagonized the effect of SGKS422D on spatial learning ($q = 3.94, p < 0.05$ comparing S422D + IKKαT23A/S180A group with the S422D group) (Fig. 8D, left). Transfection and expression of these plasmids was confirmed by immunoprecipitation and Western blot (Fig. 8D, right). In analyzing the probe trial performance of these animals, we found that SGKS422D significantly increased the time that animals spent in the target quadrant ($F_{3,31} = 3.02, p < 0.05, tD = 2.41, p < 0.05$), but this effect was blocked by IKKα double mutant cotransfection ($tD = 2.92, p < 0.01$) (Fig. 8E). We next examined the effect of knockdown of SGK1 on spatial learning. The effect...
of knockdown of Akt was also examined here. Results showed that SGK1 siRNA transfection to CA1 area significantly impaired spatial learning ($F_{2,24} = 18.15, p < 0.001; q = 4.44, p < 0.01$), but Akt siRNA markedly enhanced spatial learning ($q = 4.73, p < 0.01$) (Fig. 8F). SGK1 siRNA also markedly decreased the time that animals spent in the target quadrant for the probe.
animals were subjected to the probe trial test as described in siRNA significantly increased the time that animals spent in the probe trial test (60 s) on day 5, and the time that animals spent in each quadrant was recorded as the retention measure. Although the kinase that is regulated by SGK1 and phosphorylates IKKa at Ser-180 is not known yet, this result is consistent with the report that Ser-180 of IKKa is an important residue in regulation of IKKa activity (27). In speculation of the kinase that phosphorylates IKKa at Ser-180, few candidate proteins could be considered, for example NF-κB-inducing kinase and ERK/MAPK kinase kinase (MEKK1), because both proteins are known to phosphorylate IKKa at Ser-180 (34, 35). In addition to the role of IKKa involved in the effect of SGK1 on NF-κB activation, our results showed that IKKβ also mediates the effect of SGK1 on NF-κB phosphorylation, whereas NF-κB phosphorylation was shown to enhance the acetylation and the transcriptional activity of NF-κB (30). On the other hand, our results suggest that SGK1 does not phosphorylate IKKβ, which is inconsistent with the report showing that SGK1 phosphorylation of IKKβ at Ser-177/Ser-181 mediates the anti-apoptotic effect of SGK1 through NF-κB (25). But our results do suggest that IKKβ kinase activity is still required in mediating the effect of SGK1 on NF-κB phosphorylation and activation. These results do not conflict each other because although IKKβ was considered as the major subunit in the IKK complex to activate the NF-κB pathway, IKKa was shown to also regulate IKKβ kinase activity (36). Thus, SGK1 phosphorylation of IKKa would also activate IKKβ indirectly and consequently activate the IKK complex and the NF-κB pathway. In addition, although we have presently identified IKKa as a substrate protein of SGK1, we cannot exclude the possible involvement of other substrate proteins at a similar molecular weight. For example, the elongation factor eEF-2K has a molecular mass around 82 kDa, and it also contains the RXRXX(S/T) motif (361RvRtlS366). The identification of this protein and perhaps other proteins also as substrate of SGK1 requires further investigation.

In addition to the identified SGK1-IKKα-NF-κB pathway, we have also found that SGK1 increases the acetylation of NF-κB through phosphorylation of p300, and this pathway similarly up-regulates the expression of NR2A and NR2B. The acetylation of NF-κB is also important for NF-κB activation, and it is different from the typical IKK-mediated NF-κB activation. In this study, we have identified Ser-1834 of p300 that is phosphorylated by SGK1, and p300 phosphorylation at Ser-1834 is important for p300 acetyltransferase activity and NF-κB acetylation. This result is consistent with another report showing that p300 is phosphorylated by Akt at Ser-1834, and this phosphorylation is essential for p300 histone acetyltransferase activity (37). In another study, Hoberg et al. (38) have found that IKKa could enhance the acetylation of RelA/p65 also through p300. But these studies did not address the functional significance of these regulations. In this study, although we have found that SGK1 phosphorylates both IKKa and p300, we do not know whether these events occur in the cytoplasm or in the nucleus because total cell lysate was used for the experiments. But in another study, a nuclear role of IKKa is identified to be responsible for histone H3 phosphorylation and NF-κB activation (36). In addition, the kinase that is regulated by SGK1 and phosphorylates IKKa at Ser-180 is not known yet and also needs to be identified.

The glutamate NMDA receptor is known to play an important role in mammalian learning and memory (32). In addition, the NR2A subunit is essential for the induction of long term potentiation (39), and the NR2B transgenic mice show improved long term memory (40). How the NMDA receptor subunit has been regulated is not known. In this study, we have found that SGK1 phosphorylation of IKKa and p300 both up-regulate NMDA receptor NR2A and NR2B expression through NF-κB. Although SGK1 also increases the expression of NR1, another subunit of the NMDA receptor, our results showed that it is not regulated through NF-κB. In addition, although the promoter of the NR2B gene does not contain the known NF-κB-binding site, results from promoter activity assay revealed that there is probably an NF-κB-binding site located within nt −1480 to −20. However, it also seems that...
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FIGURE 9. A schematic diagram showing that SGK1 phosphorylation of IKKα and p300 both increase NF-κB activity and up-regulate NMDA receptor NR2A and NR2B expression. A stimulus, such as IGF-1 or insulin, would activate SGK1. SGK1 activation leads to the phosphorylation of IKKα at Thr-23 and Ser-180 that results in the phosphorylation and activation of NF-κB. SGK1 activation also phosphorylates p300 at Ser-1834 that results in the acetylation and activation of NF-κB. NF-κB activation results from these signaling events leads to up-regulation of the expression of NMDA receptor NR2A and NR2B.

the promoter sequence between nt −1000 and −1480 contains another binding element that inhibits NR2B gene expression. Future experiments are required to identify the exact location of the NF-κB-binding site on the NR2B promoter. Moreover, our result that SGK1 phosphorylation of p300 up-regulates NR2A and NR2B expression is also congruent with the report that p300 mutant mice show impaired memory performance (41).

In examination of the functional significance of the SGK1-IKKα-NF-κB signaling pathway, we have found that both IKKα phosphorylation and NF-κB activity are increased in animals subjected to water maze training and that IKKα double mutant transfection antagonized the facilitating effect of SGK1 on spatial memory formation. These results are consistent with the findings that both SGK1 and NF-κB are important for spatial learning (10, 31). They are also congruent with the reports that the IκBα double mutant and p65 knock-out mice both show impaired spatial learning and memory (31, 42). On the other hand, Akt was shown to also phosphorylate IKKα and p300 (24, 37) and up-regulate NF-κB activity (29) as does SGK1. It seems that SGK1 or Akt alone is sufficient to activate IKKα and NF-κB in terms of phosphatidylinositol 3-kinase signaling, but Akt only had a small effect in regulating NR1, NR2A, and NR2B mRNA expression. In addition, Akt siRNA treatment significantly enhanced, rather than impaired, spatial memory formation. One explanation for the opposite effect of Sgk1 siRNA and Akt siRNA on spatial memory is probably because Akt would also activate other signaling pathways that may down-regulate NR1, NR2A, and NR2B expression. Alternatively, Akt may regulate the expression of other genes, and the expression of these genes impairs spatial memory formation. However, the present results are consistent with our earlier finding that Akt transfection to the hippocampus impairs spatial learning in rats (43). The molecular mechanism underlying Akt-mediated spatial memory impairment requires further investigation.

By using an endogenous stimulus of SGK1, we have found that IGF-1 increased IKKα phosphorylation and NF-κB activity, and these effects are blocked by SGK1 siRNA treatment; yet IGF-1 did not increase NR1, NR2A, and NR2B mRNA expression. The reason for the discrepancy between these results is not known. It is possible that IGF-1 would also activate other signaling pathways that lead to the inhibition of NMDA receptor expression. In future experiments, it is worth studying whether IGF-1 also facilitates spatial memory formation and whether this effect is mediated through SGK1.

In summary, our results together suggest a novel SGK1 signaling pathway that is involved in neuronal plasticity. SGK1 directly phosphorylates IKKα at Thr-23 and indirectly activates IKKα at Ser-180. SGK1 phosphorylation of IKKα results in the phosphorylation and activation of NF-κB that consequently up-regulates NR2A and NR2B expression. In addition, SGK1 also phosphorylates p300 and that results in the acetylation, and therefore the activation, of NF-κB and enhanced expression of NR2A and NR2B (Fig. 9).

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