Regulation of Surface Localization of the Small Conductance Ca\(^{2+}\)-activated Potassium Channel, Sk2, through Direct Phosphorylation by cAMP-dependent Protein Kinase

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Small conductance, Ca\(^{2+}\)-activated voltage-independent potassium channels (SK channels) are widely expressed in diverse tissues; however, little is known about the molecular regulation of SK channel subunits. Direct alteration of ion channel subunits by kinases is a candidate mechanism for functional modulation of these channels. We find that activation of cyclic AMP-dependent protein kinase (PKA) with forskolin (50 \(\mu M\)) causes a dramatic decrease in surface localization of the SK2 channel subunit expressed in COS7 cells due to direct phosphorylation of the SK2 channel subunit. PKA phosphorylation studies using the intracellular domains of the SK2 channel subunit expressed as glutathione S-transferase fusion protein constructs showed that both the amino-terminal and carboxy-terminal regions are PKA substrates in vitro. Mutational analysis identified a single PKA phosphorylation site within the amino-terminal domain of the SK2 subunit at serine 136. Mutagenesis and mass spectrometry studies identified four PKA phosphorylation sites: Ser\(^{465}\) (minor site) and three amino acid residues Ser\(^{568}\), Ser\(^{569}\), and Ser\(^{570}\) (major sites) within the carboxy-terminal region. A mutated SK2 channel subunit, with the three contiguous serines mutated to alanines to block phosphorylation at these sites, shows no decrease in surface expression after PKA stimulation. Thus, our findings suggest that PKA phosphorylation of these three sites is necessary for PKA-mediated reorganization of SK2 surface expression.

The small conductance, Ca\(^{2+}\)-activated K\(^+\) (SK) channels are found in both neuronal and non-neuronal tissue (1). Functionally, the SK channels are best characterized in the central nervous system. Three genes encode the SK channel subunits (SK1, SK2, and SK3) in mammalian brain (2). SK channels are blocked by the bee venom toxin, apamin, although SK1 is slightly less sensitive than SK2 and SK3 (2–4). In neurons throughout the nervous system, the apamin-sensitive SK channels modulate firing frequency by contribution to the afterhyperpolarization (AHP) that follows a single or a train of action potentials (5–7). SK2 is thought to specifically underlie the medium AHP current (\(I_{m\text{AHP}}\)) in hippocampal CA1 pyramidal cells (8). The \(I_{m\text{AHP}}\) is Ca\(^{2+}\)-dependent with a time constant of 100–250 ms and sensitivity to apamin (9–11). The apamin-sensitive \(I_{m\text{AHP}}\) modulates instantaneous firing rates and sets the interspike duration in action potential trains to produce spike frequency adaptation (12). In addition, SK channels are localized to the dendrites of pyramidal cells in hippocampal area CA1 and pyramidal neurons of the lateral amygdala where they function to shape synaptic potentials and limit Ca\(^{2+}\) influx through NMDA receptors (13, 14) as well as plateau potentials evoked by exogenous glutamate application (15). Other studies have linked overexpression of SK2 and enhancement of the \(I_{m\text{AHP}}\) in hippocampus with neuroprotection (16), attenuation of hippocampal LTP, and memory deficits (16, 17). These functions have major implications for a role for SK2 channels in the formation of synaptic plasticity and learning and memory (17, 18).

In addition to the important role in pyramidal neurons, SK channels are critical for regulation of firing frequency in neurons in other parts of the nervous system. In magnocellular neurons of the hypothalamus, the characteristic bursting pattern of oxytocinergic cells that precedes milk ejection is modulated by a Ca\(^{2+}\)-dependent, apamin-sensitive \(I_{m\text{AHP}}\) that appears to be formed by SK3 channels (19, 20). Furthermore, in immature cerebellum, SK2 channels are highly expressed and determine the spontaneous firing pattern of Purkinje cells (21). In retinal ganglion cells, the apamin-sensitive \(I_{m\text{AHP}}\) that controls firing pattern and excitability is mediated by SK2 channel subunits (22). Hence, understanding the functional regulation of SK2 channel subunits would yield valuable information about the molecular regulation of the \(I_{m\text{AHP}}\).

Currently, the molecular mechanism of modulating the \(I_{m\text{AHP}}\) and neuronal firing frequency is poorly understood. A number of studies have shown that another component of the AHP current, the slow AHP current is regulated by kinase cascades (23, 24). One intriguing possible molecular mechanism of regulating the \(I_{m\text{AHP}}\) is direct kinase phosphorylation of the SK channel subunits conducting the \(I_{m\text{AHP}}\). Indeed, direct K\(^+\) channel phosphorylation is a well known post-translational modification regulating neuronal excitability. Phosphorylation of K\(^+\) channels is associated with modulation of channel kinetics and cellular localization changes (25). In addition, recent studies have suggested that activity-
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dependent potassium channel phosphorylation and functional modulation may be controlled by net kinase activity (26).

In the present study, we show that activation of PKA causes a decrease in SK2 surface expression. Furthermore, the PKA phosphorylation sites within the SK2 channel subunit were mapped, and we developed a phospho-selective antibody based on the identified sites. The phospho-selective antibody was used as a tool for further study of PKA modulation of the SK2 subunit. We showed that SK2 was phosphorylated in the COS7 cell expression system and that direct PKA phosphorylation of SK2 subunits in COS7 cells leads to altered cellular localization of the channel subunits, suggesting that the SK2 channel is a downstream target of the PKA cascade. These studies advance our understanding of the molecular regulation of SK channels by post-translational modification.

EXPERIMENTAL PROCEDURES

Molecular Biology—The original SK2 cDNA construct was kindly provided by Dr. J. P. Adelman. This construct represents the short isoform of SK2 (49 kDa). Recently a long isoform of SK2 (78 kDa) was defined with extended sequence in NT (27). For the purpose of this study, we focused on the 49-kDa isoform of the SK2 subunit. Plasmids containing the NT (amino acid residues 1–140), CT (residues 395–580), and SK2 subunit second intracellular loop (residues 278–306) domains were constructed using the modified glutathione S-transferase (GST) fusion vector pGEX-YR with NotI and EcoRI sites. Site-directed mutagenesis was performed on the 49-kDa isoform of the SK2 subunit. Plasmids were transformed with plasmids containing the NT, CT, or second intracellular loop domains were selected and grown in LB broth. After growing to an optical density of 0.6–0.8 (A590), the bacteria were induced by incubation at room temperature with 200 μM isopropyl β-d-thiogalactopyranoside (Sigma) for 4 h and were harvested by centrifugation at 3,000 × g for 10 min. The cells were resuspended and incubated in STE buffer (in mM): 10 Tris-HCl, pH 8.0, 1 EDTA, 150 NaCl, containing Protease Inhibitor Mixture (Sigma) and 100 μg/ml lysozyme (Sigma) for 15 min on ice. 10 mM dithiothreitol and 1.5% N-lauroylsarcosine were added, and then the sample was sonicated for a total of 2 min. The lysates were then centrifuged (10,000 × g, 20 min, 4 °C) and adjusted to 0.7% N-lauroylsarcosine and 2% Triton X-100. The GST fusion proteins were purified using glutathione affinity absorption. Glutathione-agarose beads (Amersham Biosciences) were washed, resuspended in phosphate-buffered saline (PBS, pH 7.4; in mM: 137 NaCl, 2.7 KCl, 4.3 Na2HPO4, 1.4 KH2PO4) and then incubated with the lysates for 1 h at room temperature or overnight at 4 °C. The beads were washed three times with PBS buffer by centrifugation (100 × g, 5 min, 4 °C). After the final wash, the bead preparation was resuspended in PBS buffer containing Protease Inhibitor Mixture. The recombinant proteins were left on the beads for subsequent experiments and stored at 4 °C.

PKA Phosphorylation of GST SK2 Fusion Proteins—GST SK2-NT or -CT fusion proteins were incubated for 30 min at 37 °C in reaction mixtures (50 μl) containing 70 ng of the catalytic subunit of PKA (Sigma), Tris buffer, and ATP mix buffer (100 μM ATP, 100 mM MgCl2, and 10 μCi of[y-32P]ATP). For time-course studies of phosphorylation of the GST SK2-NT and -CT fusion proteins incubation conditions for 5, 10, 30, 60, and 90 min were used. Reactions were stopped by boiling for 5 min at 95 °C with sample buffer (30 mM Tris-HCl, pH 6.8, 200 mM dithiothreitol, 40% glycerol, 8% SDS, 0.04 mg/ml bromphenol blue). The GST fusion proteins were separated by 12.5% SDS-PAGE and visualized by Coomassie Blue staining. Phosphopeptides were identified by autoradiography. As a control, parallel reactions were performed for GST alone and PKA alone with ATP mix buffer. The optical density of autoradiography was normalized to the corresponding Coomassie bands (29).

Phosphopeptide Mapping—Identification of the specific amino acids phosphorylated by PKA within the SK2 CT was performed by a modified tandem mass spectroscopy method in the Protein Chemistry Core Facility at Baylor College of Medicine as previously described (30). The production of a 60-min preparative scale reaction (volume of 300–400 μl) of activated PKA, the GST SK2-CT, and ATP was separated by SDS-PAGE (12.5%) stained by Coomassie Blue and digested in-gel by Lys-C. The separated peptide fragments were desalted using a C18 ZipTip column (Millipore) then analyzed by electrospray ionization mass spectroscopy (API 3000 LC/MS/MS System, PE Scieix, Thornhill, Ontario, Canada). Phosphopeptides were identified by a −79-dalton precursor ion scan and subsequently sequenced with tandem mass spectrometry.
Phospho-antibody Production—Synthetic peptides corresponding to SK2 residues 558–580, which included the identified phosphorylation sites (Ser568, Ser569, and Ser570, unphosphorylated and phosphorylated) were produced by the Protein Chemistry Core Facility at Baylor College of Medicine. The peptides were synthesized with a cysteine at the C-terminal end of the peptide to allow conjugation to the carrier protein keyhole limpet hemocyanin via m-maleimidobenzoyl-N-hydroxysuccinimide ester. The conjugates of the phosphopeptides were used for the production of custom antibodies (Cocalico, Inc., CA). After a pre-immune blood sample was taken, the conjugates of the phosphopeptide were injected into two albino New Zealand rabbits with the adjuvant Titermax via intradermal injections. The animals were boosted with antigen several times. The phospho-SK2 antisera was affinity-purified and characterized with Western blotting (28, 29).

COS7 Cell Expression Systems and Membrane Preparation—COS7 cells were cultured and transfected with FuGene6 (Roche Diagnostics). In brief, cells were grown in poly-D-lysine-coated 6-well plates and maintained in Dulbecco’s modified eagle’s medium (Invitrogen) containing fetal bovine serum and penicillin/streptomycin. 1% serum was used in all subsequent PKA manipulation experiments. Transfection of the SK2 channel was performed on COS7 cells with FuGENE 6 and grown for 36–48 h. The media was switched from 10% serum to 1% serum 12 h before PKA manipulation. Sham transfections were performed using a blank vector (pDs-Red, Clontech, Palo Alto, CA) or a Kv4.2 construct. COS7 cells were treated with PKA modulators dissolved in Me2SO. Me2SO alone served as the vehicle control. In all conditions the final concentration of Me2SO was 0.2%. The PKA pathway manipulations were are follows: 1) Me2SO alone; 2) 15-min incubation with 50 μM forskolin (FSK), PKA activator plus 100 μM Ro-201724 (phosphodiesterase inhibitor) (28); 3) 30-min preincubation with 10 μM H89 (PKA inhibitor) (31); or 4) preincubated with 10 μM H89 for 30 min followed by a 15-min incubation with 50 μM FSK/100 μM Ro-201724. Cell membrane preparation was performed; briefly, plates were put on ice to stop the reaction and washed three times with cold PBS with phenylmethylsulfonyl fluoride (PMSF, 100 μM). After harvesting by scraping cells off the plates in BHB buffer (in mM: 20 Tris, pH 7.5, 1 EGTA, 1 EDTA, 1 Na2HPO4, 1 Na2VO4, 0.1 PMSF), membranes were prepared by centrifuging at 92,500 × g at 4 °C for 60 min. The cell pellet was resuspended in 5% SDS with 100 mM dithiothreitol, 100 μg/ml pepstatin, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 100 μM PMSF. Sample buffer was then added, and the samples were loaded on a SDS-PAGE gel for Western blotting.

Immunostaining of COS7 Cells—Transfected COS7 cells were treated with the PKA activators or inhibitors and then fixed with 4% paraformaldehyde (Polysciences, Inc., Warrington, PA) for 40 min at room temperature. Cells were rinsed twice with PBS and treated with 0.3% Triton X-100 for 15 min at room temperature. The cells were blocked with 10% fetal bovine serum for 1 h at room temperature followed by SK2 antibody diluted 1:500 in PBS overnight at 4 °C. The cells were then washed in 0.1% Triton X-100 or Tween 20 three times for 5 min each. Alexa Fluor 488 goat anti-rabbit IgG (H+L) secondary antibody (Molecular Probes, Eugene, OR) was diluted to 1:5000 and incubated with cells for 30–40 min at room temperature. The cells were visualized on the
FIGURE 2. Candidate PKA consensus sites within the SK2 channel subunit. The amino acid sequence of the SK2 channel subunit is shown with the intracellular regions underlined. The full-length SK2 subunit consists of 580 amino acid residues with a putative transmembrane domain of six transmembrane regions, two intracellular loops, and cytoplasmic amino- and carboxyl-terminal domains. We inspected the primary amino acid sequence of the intracellular domains of the SK2 subunit for known PKA consensus sequences (RxRXS/TDXD) as these regions may be accessible to intracellular PKA. Seven amino acid residues (Ser15, Ser16, Thr19, Ser36, Thr44, Ser45, and Ser208) were predicted as candidate PKA phosphorylation sites within SK2 by NetPhos (www.cbs.dtu.dk/services/NetPhos/). The predicted sites are indicated in bold and are numbered.

Nikon TE-200 inverted fluorescence microscope. All immunohistochemistry studies were performed in three independent experiments.

**Cell-surface Biotinylation**—After PKA pathway manipulation, the SK2-transfected COS7 cells were washed with ice-cold PBS containing 0.1 mM CaCl2 and 1 mM MgCl2 (PBS/Ca2+/Mg2+) plus Mg2+/Ca2+ (pH 8.0) then treated with sulfo-NHS-SS-biotin (0.5 mg/ml, Pierce) at 4 °C for 40 min. Cells were lysed in radioimmuno precipitation assay buffer (150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 20 mM NaHPO4, pH 7.4), supplemented with 0.01 mM PMSF, 0.005 μg/ml leupeptin, and 0.005 μg/ml pepstatin at 4 °C. Lysates were centrifuged at 20,000 × g for 30 min at 4 °C, and the protein concentration in the supernatants was determined using the BCA assay kit (Pierce). UltraLink Immobilized NeutrAvidin beads (50 μl, Pierce) were added to each group sample, and the mixture was incubated for 1 h at room temperature or 4 °C overnight. The beads were washed four times with cold radioimmuno precipitation assay buffer and eluted with 50 μl of Laemmli loading buffer (Bio-Rad) for 5 min at 95 °C. The eluates (25 μl) were resolved by SDS-PAGE gel and immunoblotted with the commercial SK2 antibody. Immunoreactive bands were visualized using horseradish peroxidase-conjugated secondary antibody (1:10,000). Immunoreactivity values of surface SK2 channels were normalized to levels of actin immunoreactivity in total cell extracts to preclude errors that accompany sample loading and transfer.

**Western Blot Analysis**—Gels were blotted electrophoretically to Immobilon filter paper using a transfer tank maintained at 4 °C as described previously (32). Blots were blocked for 1 h at room temperature in a blocking solution containing 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20, 5% milk, 0.1% thimerosal. The blots were incubated at room temperature with one of the following primary antibodies: total SK2 antibody, 1:500, Alomone Labs, Jerusalem, Israel; phospho-SK2 antibody, 1:500, phospho-PKA substrate antibody, 1:1000 (Cell Signaling Technology, Beverly, MA) for 1 h. After washing, blots were incubated in a horseradish peroxidase-conjugated secondary antibody (1:20,000, Cell Signaling Technology) for 60 min. Blots were washed extensively in TTBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) and then exposed to enhanced chemiluminescence (ECL) or SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology, IL) was used for detection of immunoreactivity.

**Data Analysis**—Immunoreactivity was quantified by densitometry (Scion Corp., version Beta4). The GraphPad Prism software package was used for statistical analysis of the data. Data are expressed as standard error of mean (S.E.). Representative examples are shown under “Results.” One-way analysis of variance with post-hoc analysis or Student’s t test was used for comparison. Statistical significance was taken as p < 0.05.

**RESULTS**

The PKA Pathway Modulates SK2 Channel Surface Expression in COS7 Cells—We first investigated PKA pathway regulation of SK2 channel trafficking and localization in the COS7 cell expression system. Immunostaining was performed on the SK2-transfected COS7 cells with the commercial SK2 antibody after treating the cells with PKA pathway modulators (Fig. 1A). Immunostaining for SK2 was visualized on the cell-surface membrane and concentrated in presumptive endoplasmic reticulum (ER) and Golgi apparatus in the vehicle control (Me2SO). PKA activation using forskolin (FSK (50 μM)) together with the phosphodiesterase inhibitor Ro-201724 (100 μM) attenuated surface expression and increased perinuclear expression (presumptive ER and Golgi complex) of the SK2 subunits, whereas the PKA inhibitor, H89 (10 μM), facilitated SK2 channel trafficking to the cell surface. The decrease in surface expression of SK2 channels by forskolin is attenuated by preincubation with H89. These data suggest that PKA regulates the surface expression of SK2. As controls and in parallel with the studies presented above, we processed untransfected and sham-transfected COS7 cells for SK2 immunohistochemistry. These COS7 cells showed no SK2 staining (sham-transfected COS7 cells shown in supplemental Fig. S1).

We further investigated this effect with surface biotinylation experiments to quantify the cell-surface expression of SK2 following PKA cascade manipulation in COS7 cells. Western blotting with the commercial SK2 antibody was performed to compare the biotinylated and total pools of SK2 channels in each experimental group (Fig. 1B). Western blotting showed no difference in the total SK2 channel expression in lysates of all treated groups. However, there was a significant reduction in SK2 channel expression on the cell surface following forskolin application relative to control (65 ± 4% of Me2SO control, p < 0.05, n = 3) (Fig. 1C). Western blotting with a second SK2 antibody (generated in the laboratory of J.P.A.) against a different epitope (SK2 residues 538–547) revealed similar results (data not shown). In contrast, following H89 treatment, there was a significant elevation of SK2 channel expression on the cell surface compared with control (168 ± 13% of Me2SO control, p < 0.01, n = 3). There was no significant difference in surface expression of cells treated with both FSK and H89 (104 ± 9% of Me2SO control). Together, these results demonstrate that, in SK2-transfected COS7 cells, activation of the PKA cascade attenuates surface expression of SK2 channel subunits, whereas a reduction of PKA activity increases SK2 subunit surface expression. Based on the observation that there are
PKA consensus sites within the SK2 amino acid sequence, we hypothesized that PKA regulates SK2 surface expression by direct phosphorylation of the SK2 subunits.

Candidate PKA Sites within the SK2 Amino Acid Sequence—The SK2 subunit is composed of 580 amino acid residues with six putative transmembrane domains, and cytoplasmic NT and CT regions. We focused our study on SK2 cytoplasmic domains, because these would be accessible to cellular kinases. Analysis of the amino acid sequence of SK2 with NetPhos (www.cbs.dtu.dk/services/NetPhos/) (33) predicted multiple serine and threonine residues (>95% probability) as candidate PKA phosphorylation sites. Two candidate PKA sites were located within the NT and five within the CT cytoplasmic domains (Fig. 2). No candidate PKA sites were identified within the two cytoplasmic loops.

PKA Phosphorylates the SK2-NT and -CT Domains—To test our hypothesis that the SK2-NT and -CT domains are PKA substrates, recombinant GST fusion proteins of SK2-NT (amino acid residues 1–140) and -CT (amino acid residues 395–580) regions were incubated with the PKA catalytic subunit and [γ-32P]ATP in vitro. Reaction products were separated using SDS-PAGE and visualized by Coomassie Blue staining (Coomassie) and autoradiography (Autorad). The initial reactions were performed using an incubation period of 30 min (A), and subsequently a time course of the phosphorylation events was performed (B). Coomassie Blue-stained gels demonstrate bands at ~42 kDa (GST SK2-NT) and at 50 kDa (GST SK2-CT) representing the NT and CT constructs, respectively (left panel). The autoradiogram shows 32P incorporation into the GST SK2-NT and -CT constructs, respectively (right panel), suggesting that both the SK2-NT and -CT domains are PKA substrates. Note that GST alone (28 kDa) and PKA alone (without peptide substrates) were not phosphorylated by PKA in vitro, B. The time course of phosphorylation at Ser136 is the only amino acid residue phosphorylated by PKA within the SK2-NT region.
Mapping of the PKA Phosphorylation Sites within the SK2-CT Domain—A number of potential PKA phosphorylation sites were predicted within the SK2-CT cytoplasmic domain; hence, we initially used mass spectrometry (MS) to map phosphorylation sites. The PKA-phosphorylated GST SK2-CT fusion protein was digested with Lys-C, desalted on a resin column, and analyzed by matrix-assisted laser desorption ionization (MALDI) scanning in tandem with negative ion precursor scanning. MALDI was able to identify peptides containing three of the five potential PKA sites: 430ETWLYK436, 455KFLQAIHQIRSVK467, and 507RIVTTEK514 with the predicted PKA sites Thr431, Ser465, and Thr510 (predicted PKA sites), but these peptides were not phosphorylated.

FIGURE 5. PKA phosphorylates the SK2 carboxyl-terminal cytoplasmic domain. A, to map the PKA phosphorylation sites within the SK2-CT domain we performed a large scale phosphorylation reaction with the PKA catalytic subunit and the GST SK2-CT construct. The reaction product was digested in-gel by Lys-C and analyzed with tandem electrospray ionization mass spectroscopy for peptide-associated phosphate groups. A - 79 precursor ion scan and tandem mass spectroscopy revealed that a phosphate group was associated with a peptide corresponding to SK2 residues 455KFLQAIHQIRSVK467 with the m/z ratio of 823.0 (arrow). These results indicate that Ser465 within the GST SK2-CT construct is phosphorylated by PKA. Mass spectroscopy also identified peptides containing SK2 amino acid residues Thr431 and Thr510 (predicted PKA sites), but these peptides were not phosphorylated. B, we evaluated phosphorylation of GST SK2-CT wild-type compared with a S465A GST SK2-CT mutant construct. Coomassie Blue (Coomassie) staining shows 49-kDa bands, representing the GST SK2-CT wild-type (WT) and mutant (S465A) constructs. Autoradiography (Autorad) demonstrates robust 32P incorporation into both of the constructs. These findings suggest that Ser465 may be a minor site and that there are additional phosphorylation sites within the SK2 CT. C, serine to alanine mutants of the candidate PKA sites, Ser465 and Ser565 in combination with Ser465 within the GST SK2-CT construct were tested for PKA phosphorylation. Coomassie Blue staining and autoradiography show 32P incorporation into GST SK2-CT mutants: S561A, S568A, S465A/S561A, S465A/S568A, and S465A/S561A/S568A. These data suggest that there are additional PKA phosphorylation sites located within the SK2-CT region.
PKA. Thus, these findings suggest that Ser<sup>465</sup> but not Thr<sup>431</sup> and Thr<sup>510</sup> is a PKA phosphorylation site within the SK2-CT. Peptides containing the two other predicted PKA sites, Ser<sup>561</sup> and Ser<sup>568</sup>, were not obtained on MALDI scanning, possibly because the Lys-C digest yielded small peptide fragments that were not detected. Digests obtained with other proteases such as pepsin and Glu-C were also unsuccessful at yielding peptides containing these amino acids (data not shown). Hence, we were unable to evaluate PKA phosphorylation at these sites (Ser<sup>561</sup> and Ser<sup>568</sup>) using this technique.

As an alternative approach, we used site-directed mutagenesis to determine if the remaining predicted sites were phosphorylated by PKA. Site-directed mutation of serine to alanine at position 465, to remove the PKA phosphorylation site did not decrease <sup>32</sup>P incorporation into the GST SK2-CT construct (Fig. 5B). These data suggest that additional PKA phosphorylation sites exist within the SK2-CT region, possibly in the serine- and arginine-rich CT tail. To identify the remaining PKA phosphorylation site(s), GST SK2-CT constructs with mutations of the predicted Ser<sup>561</sup> and Ser<sup>568</sup> sites and the mapped S<sup>465</sup> site were generated and incubated with activated PKA and [γ-<sup>32</sup>P]ATP in vitro (Fig. 5C). Phosphorylation was still observed in these constructs. These findings suggest that additional PKA phosphorylation sites within the SK2-CT domain are present. In addition, these data also suggest that Ser<sup>465</sup> is a minor phosphorylation site.

We hypothesized that the additional PKA sites reside in the serine- and arginine-rich distal SK2-CT tail. Thus, we assessed <sup>32</sup>P incorporation within the GST SK2-CT wild-type compared with three truncated GST SK2-CT fusion proteins, S<sup>559</sup>stop, S<sup>568</sup>stop, and T<sup>575</sup>stop following incubation with activated PKA (Fig. 6A, upper panels). The <sup>32</sup>P incorporation was found within the GST SK2-CT wild-type and T<sup>575</sup>stop construct but not within the S<sup>559</sup>stop and S<sup>568</sup>stop constructs (Fig. 6A, lower panels). These findings suggest that additional PKA phosphorylation site(s) are located distal to residue 567 within the SK2-CT tail. To further define the PKA phosphorylation sites within this region, we mutated residues S<sup>568</sup>S<sup>571</sup> to AAAAA within the GST SK2-CT construct (Fig. 6B, upper panel number 2) and performed PKA phosphorylation reactions with [γ-<sup>32</sup>P]ATP. No <sup>32</sup>P incorporation was found within the GST SK2-CT S<sup>568</sup>AAAA<sup>571</sup> construct (Fig. 6B, lower panel, lane 2), suggesting that the major phosphorylation sites were restricted to the S<sup>568</sup>S<sup>571</sup> region. A series of serine to alanine mutations were made within the S<sup>568</sup>S<sup>571</sup> region to identify the specific residues phosphorylated by PKA (Fig. 6B, upper panel). There was <sup>32</sup>P incorporation within the SK2-CT S<sup>568</sup>S<sup>571</sup>, S<sup>568</sup>A<sup>571</sup>, and S<sup>568</sup>AAAA<sup>571</sup> constructs but not in S<sup>568</sup>A<sup>569</sup>A<sup>571</sup> construct (Fig. 6B, lower panels, lanes 3–6). These findings suggest that the residues Ser<sup>568</sup>, Ser<sup>569</sup>, and Ser<sup>570</sup> within SK2 are PKA substrates.

A summary of all of the PKA sites that we mapped within the SK2 cytoplasmic domain are presented in a model diagram of the SK2 channel (Fig. 7). As indicated, only one phosphate site was identified within the SK2-NT domain, while four additional sites were identified within the SK2-CT domain. For the studies testing the functional significance of direct phosphorylation of the SK2 channel by PKA presented below, we focused on the three sites (Ser<sup>568</sup>, Ser<sup>569</sup>, and Ser<sup>570</sup>) located within the distal SK2-CT domain. This was based on two observations: 1) Ser<sup>568</sup> within the NT domain of SK2 resides at the beginning of the first transmembrane domain, suggesting that this residue may not be accessible to intracellular kinases in the full-length channel, and 2) Ser<sup>465</sup> appears to be a minor phosphorylation site based on our studies using the GST SK2-CT construct (see Fig. 5B).

PKA Phosphorylates Full-length SK2 Channel Subunits in the COS7 Expression System—Because our results showed that PKA phosphorylates SK2 in vitro, we hypothesized that SK2 is phosphorylated by PKA in a cell system. We generated a phospho-selective antibody against a synthetic peptide containing phosphorylated residues Ser<sup>465</sup>, Ser<sup>469</sup>, and Ser<sup>570</sup> within the SK2-CT region that was blocked by preincubation with the antigenic peptide (0.26 nM, Fig. 8C). These initial screening studies suggest that the antibody is phospho-selective.

The antibody was used to assess PKA phosphorylation of the full-length SK2 channel subunit in the COS7 expression system. COS7 cells transiently expressing wild-type SK2 were incubated with forskolin (50 μM, FSK, together with 100 μM Ro-201724) or H89 (10 μM) for PKA activation or inhibition, respectively, of the endogenous PKA cascade. Membranes were
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FIGURE 7. Summary of the PKA phosphorylation sites within the SK2 channel subunit. A: model diagram of the SK2 channel is shown with putative six transmembrane domains and the intracellular amino- and carboxyl-terminal (NT and CT, respectively) domains. Five PKA phosphorylation sites were identified within intracellular domains: Serine (S) 136 within the NT and Ser465, Ser568, Ser569, and Ser570 within the CT. The Ser136 site lies within a region of the SK2 that is at the junction of the first transmembrane domain. Ser465 is located in the proximal CT, whereas the Ser568, Ser569, and Ser570 sites are located in the distal tail of the CT cytoplasmic domain. The PKA phosphorylation sites are represented by open circles.

FIGURE 8. Development and screening of the phospho-selective SK2 antisera. To measure PKA phosphorylation of the SK2 subunits, phospho-selective antisera were generated and the affinity-purified antibody was screened by Western blotted. A: sequences of synthetic SK2 CT peptides. B: immunoreactivity of the phospho-SK2 antibody. C: antigenicity of the phospho-SK2 antibody. Preincubation of the anti-SK2 antibody with the unphosphorylated synthetic peptide or fusion protein (Fig. 8, left panels) to remove phosphates was blocked by preincubation of the antigenic peptide (Pep) in a concentration-dependent manner (lanes labeled Pep (2.6 nM), Pep (0.26 nM), and Pep (0.1 nM)). C, phospho-selectivity of the affinity-purified antibody was screened by Western blotting using the unphospho- and phospho-SK2-CT GST construct. The left panel (Ab) shows antibody recognition of the phospho-SK2-CT GST construct (lane 1) with no recognition of the unphospho-SK2-CT GST fusion protein (lane 2). In parallel studies immunoreactivity of the phospho-antibody to the phospho-SK2-CT GST construct was blocked by preincubation of the antibody with the phospho-peptide antigen (right panel, Ab + Pep (0.26 nM)).

Prepared from SK2-transfected COS7 cells after PKA pathway manipulations. Western blots were probed with the phospho-SK2 antibody and a commercial SK2 antibody that is not sensitive to the phosphorylation state of SK2 (same antibody used in Fig. 1 studies). Actin immunoreactivity was used for normalization. Densitometry revealed a significant increase in immunoreactivity with the phospho-SK2 antibody following forskolin stimulation compared with vehicle (Me$_2$SO)-treated control COS7 cells (259 ± 21% of control, p < 0.001, n = 4). This effect was blocked by preincubation with H89 (H89 plus FSK, 149 ± 16% of control, not significant compared with control, compared with FSK, p < 0.001, n = 4) (Fig. 9A, left panels). There was no significant change in immunoreactivity using the commercial SK2 antibody following PKA pathway manipulation (Fig. 9A, right panels), indicating that this manipulation does not result in a change in SK2 protein expression. No immunoreactivity was seen in lanes containing membranes from sham-transfected COS7 cells treated with vehicle (Sham) or forskolin (Sham plus FSK) that were probed with the phospho-SK2 or commercial SK2 antibodies (Fig. 9A). Based on this series of experiments, we conclude that PKA activation couples to direct phosphorylation of residues Ser568, Ser569, and Ser570 within the SK2 channel subunit.

The Me$_2$SO-treated SK2-transfected COS7 cultures demonstrated basal levels of immunoreactivity with the phospho-SK2 antibody, suggesting that there are basal levels of SK2 phosphorylation, potentially explaining the increase in surface expression produced by H89 treatment (Fig. 1). However, another possibility is that the antibody has some recognition of the unphosphorylated SK2 channel. We considered the latter possibility unlikely, because the antibody was affinity-purified against the phospho-SK2 peptide used to make the antibody and the initial screening of the phospho-SK2 antibody did not reveal detection of the unphosphorylated synthetic peptide or fusion protein (Fig. 8, B and C). However, to confirm the specificity of the phospho-SK2 antibody, alkaline phosphatase (AP, 5 units/µl) to remove phosphates was incubated with SK2-transfected COS7 lysates after PKA pathway manipulations. An aliquot of the lysate from each condition was incubated in parallel to the AP-treated samples for comparison. Western blotting of the lysates was performed using the phospho-SK2 antibody. In addition, we used an antibody that recognizes a wide variety of PKA phosphorylated substrates. As expected, PKA stimulation with forskolin and inhibition with H89 lead to modulation of the immunoreactivity of a 49-kDa band corresponding to SK2 in Western blotting with the phospho-SK2 antibody (Fig. 9B, top panel). The PKA substrate antibody also detected modulation of the immunoreactivity of several bands of varying molecular weight (Fig. 9B, middle panel). Alkaline phosphatase treatment abolished immunoreactivity for both the phospho-SK2 and PKA substrate antibodies. There was no change in total actin levels. These findings indicate that the antibody is phospho-selective and suggest that there are basal levels of SK2 phosphorylation in COS7 cells transiently expressing SK2. As an additional control for the SK2 anti-
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The studies presented here demonstrate that the PKA pathway regulates surface expression of SK2 homomeric channels in the COS7 expression system. PKA activation decreased SK2 surface localization, whereas PKA inhibition had the opposite effect. To determine the mechanisms of this effect, we further investigated whether SK2 was directly phosphorylated by PKA and whether this phosphorylation was responsible for the effect. Through a series of mapping and surface biotinylation experiments, residues Ser$^{568}$, Ser$^{569}$, and Ser$^{570}$ within the distal CT of the SK2 cytoplasmic domain were identified as the PKA phosphorylation sites responsible for the trafficking effect of PKA activation on SK2 surface expression. We also showed that PKA activation increases phosphorylation of expressed SK2 channel subunits at these sites in COS7 cells using a phospho-selective antibody. Furthermore, biotinylation and immunostaining studies showed a decrease in surface expression of SK2 following activation of the endogenous PKA cascade. Modulation of SK2 channel surface expression by PKA was eliminated in the SK2 construct with serine to alanine mutation of the three PKA sites within the distal CT tail of SK2, and PKA inhibition had the opposite effect. To determine the PKA effect is related to phosphorylation of these serines, we performed PKA activation experiments in SK2 channel subunits transfected with full-length or wild-type SK2 channel subunits with Ser$^{568}$, Ser$^{569}$, and Ser$^{570}$ residues mutated to alanine (SK2-AAA). A, following PKA manipulations, PKA phosphorylation of SK2 WT in transfected COS7 cells was measured by Western blotting membrane fractions using the phospho-SK2 antibody (P-SK2, left panel). PKA activation with forskolin (FSK; 50 μM) together with 100 μM Ro-20-1724, a phosphodiesterase inhibitor, significantly increased SK2 phosphorylation relative to vehicle (0.2% Me2SO) control (259 ± 21% of control; *** p < 0.001), which was significantly attenuated by PKA inhibition with H89 preincubation (10 μM, H89 + FSK) (49 ± 16% control, compared with FSK; **, p < 0.001). Phospho-SK2 immunoreactivity following preincubation with H89 alone (H89) was unchanged compared with control. In parallel studies, blots probed with the commercial SK2 antibody (not sensitive to phosphorylation sites) showed no differences in SK2 expression (SK2, right panels), since there were basal levels of immunoreactivity in the control and H89 conditions, we evaluated whether the affinity-purified phospho-SK2 antibody recognized unphosphorylated channel proteins. Aliquots of SK2 WT-transfected COS7 cells treated with PKA pathway modulators were incubated with or without alkaline phosphatase (AP, 5 unit/μl). Representative Western blots of total lysates probed with the phospho-SK2 (P-SK2), a commercial phospho-PKA substrate (PKA substrate), or actin (Actin) antibody are shown. Treatment of the samples with AP following PKA manipulation (AP treated) eliminated immunoreactivity with the phospho-SK2 antibody. Probing the blots with the actin antibody showed equal protein loading across lanes. Blots probed with the PKA substrate antibody showed that there was an increase in phosphorylated proteins following PKA activation (FSK) compared with basal levels in the control (Me2SO, DMSO), H89 + FSK reduced the increase in PKA substrate phosphorylation following FSK only. In parallel experiments, application of AP to the samples eliminated the immunoreactivity seen with the PKA substrate antibody. C, as an additional confirmation of the phospho-selectivity of the P-SK2 antibody, we performed PKA activation experiments in COS7 cells expressing the full-length SK2-AAA mutant channel subunit. Western blotting with the P-SK2 antibody following PKA cascade activation (FSK) compared with control (Me2SO, DMSO) demonstrated no immunoreactivity with whole cell lysates (top panel). Parallel lysates probed with the SK2 antibody (not phospho-selective) demonstrated that the SK2-AAA subunit was expressed (bottom panel). Data are expressed as mean ± S.E. One-way analysis of variance with post-hoc analysis was used for comparison, n = 4.

The studies presented here demonstrate that the PKA pathway regulates surface expression of SK2 homomeric channels in the COS7 expression system. PKA activation decreased SK2 surface localization, whereas PKA inhibition had the opposite effect. To determine the mechanisms of this effect, we further investigated whether SK2 was directly phosphorylated by PKA and whether this phosphorylation was responsible for the effect. Through a series of mapping and surface biotinylation experiments, residues Ser$^{568}$, Ser$^{569}$, and Ser$^{570}$ within the distal CT of the SK2 cytoplasmic domain were identified as the PKA phosphorylation sites responsible for the trafficking effect of PKA activation on SK2 surface expression. We also showed that PKA activation increases phosphorylation of expressed SK2 channel subunits at these sites in COS7 cells using a phospho-selective antibody. Furthermore, biotinylation and immunostaining studies showed a decrease in surface expression of SK2 following activation of the endogenous PKA cascade. Modulation of SK2 channel surface expression by PKA was eliminated in the SK2 construct with serine to alanine mutation of the three PKA sites within the distal CT tail of SK2. These results support an important role for these sites in PKA-mediated alterations in localization and trafficking of the SK2 channel subunit. The functional significance of PKA phospho-regulation at Ser$^{568}$, Ser$^{569}$, and Ser$^{570}$ sites in the full-length SK2 subunit in a cell system remains to be shown.

Ion channel trafficking involves the secretory and endocytic pathways to and from the surface membrane and the regulatory elements in this process are diverse. Recently it has been shown that the activity of protein kinase pathways is an important determinant of potassium channel trafficking (34). Our findings demonstrate that PKA activation leads to a decrease in SK2 channel subunit expression at the COS7 cell surface through direct phosphorylation of the SK2 subunits. Kinase regulation of SK2 surface expression has not previously been shown; however, SK2 association with calmodulin is obligatory for SK2 surface expression in COS7 cells (35). It is possible that phosphorylation of the sites that we mapped leads to conformation changes that disrupt calmodulin binding. It is unlikely that the PKA effect is related to phosphorylation of
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PKA Phosphorylation of the SK2 Channel Subunit Suggests Kinase Action

PKA phosphorylation of the SK2 channel subunit is critical for PKA-induced down-regulation of SK2 surface expression. Data normalized to actin. These findings suggest that contiguous phosphorylation sites face not sensitive to the phosphorylation state of SK2. Representative blots of surface (near the PKA sites within the distal CT tail of the SK2 subunit, corresponding R type ER retention signal (42). There are three RX sequences found in ion channels, which are critical for PKA-induced down-regulation of SK2 surface expression. Data are expressed as mean ± S.E. Student’s paired t test was used for analysis.

calmodulin, because there are no PKA consensus sequences within the calmodulin amino acid sequence.

Direct phosphorylation of ion channels or auxiliary subunits contributes to protein trafficking to the surface membrane (36, 37). However, a number of studies have shown that ER retention signals also play an important role in the trafficking of membrane proteins to the cell surface (38 – 40). The ER retention signals, particularly the RXR sequence in ion channels, are found mainly at the extreme CT of proteins. These amino acid sequences function to retain newly formed proteins in the ER and allow for retrieval from the Golgi apparatus. The retention signals become masked to ensure that only completely assembled channels are delivered to the surface membrane (38). In addition, Zhou et al. (41) reported that both phosphorylation and RXR type ER retention signals are required for PKA-mediated potentiation of sodium currents in heart. It also has been reported that trafficking of the Kir1.1 channel to the surface membrane by direct phosphorylation overrides the independent RXR type ER retention signal (42).

The effects of PKA phosphorylation of SK2 in excitatory tissue where the channels are natively expressed have not been elucidated; however, our findings suggest a molecular locus for previous reports of the regulation of the I_{mAHP} which is thought to be formed at least in part, by SK2 channels (18). The enhancement of theta-burst LTP induction in hippocampal slices by brain-derived neurotrophic factor (BDNF) is associated with an increase in direct serine phosphorylation within SK2 and a reduction in the I_{mAHP} (43). The specific kinase that is activated and serines that are phosphorylated within SK2 in BDNF-enhanced theta-burst LTP remain to be elucidated. However, BDNF transiently activates PKA in the hippocampus (44). Thus, SK2 may be a direct target of the PKA cascade in BDNF-modulated neuronal plasticity. Moreover, functional studies in rat jaw-closing spinal motor neurons have coupled activation of the metabotropic 5-HT_{1A} receptor to down-regulation of the aminergic-sensitive I_{mAHP} (45). The exact pathway mediating this effect in spinal motor neurons is unknown, but activation of the 5-HT_{1A} receptor induces downstream CAMP accumulation and PKA activation (46). Therefore, activation of 5-HT_{1A} receptors in spinal motor neurons may result in direct phosphorylation of the SK2 subunit and modulation of the I_{mAHP}.

The phospho-selective antibody that we have generated may provide a tool to assess PKA phosphorylation of SK2 in cell types that natively express SK2 channels. In preliminary studies we have begun to evaluate PKA pathway coupling to SK2 phosphorylation in hippocampal tissue.

In these studies, the phospho-SK2 antibody recognizes a band at roughly 49 kDa that increases with PKA pathway activation by forskolin. The commercial SK2 antibody recognizes a band at the same molecular weight that does not modulate with forskolin stimulation. These findings suggest that PKA phosphorylation of SK2 occurs in a physiologically relevant system and that the phospho-antibody is selective in a tissue type where SK2 channels are natively expressed.

In conclusion, we have demonstrated that direct PKA phosphorylation of SK2 regulates the localization of SK2 channel proteins in the COS7 expression system. The altered cellular distribution of SK2 channels may play a role in the modulation of the I_{mAHP} and regulate excitability in tissues where the channels are expressed, such as the nervous system. Given the recently defined role of SK2 in regulation of NMDA receptor-mediated synaptic potentials and Ca^{2+} influx (13, 14), modulation of SK2 surface expression by PKA phosphorylation may have dramatic consequences on cellular plasticity and learning and memory.

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