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The neuronal-specific SGK1.1 kinase regulates δ-epithelial Na^+ channel independently of PY motifs and couples it to phospholipase C signaling

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Wesch D, Miranda P, Afonso-Oramas D, Althaus M, Castro-Hernández J, Dominguez J, Morty RE, Clauss W, González-Hernández T, Alvarez de la Rosa D, Giraldez T. The neuronal-specific SGK1.1 kinase regulates δ-epithelial Na^+ channel independently of PY motifs and couples it to phospholipase C signaling. *Am J Physiol Cell Physiol* 299: C779–C790, 2010. First published July 14, 2010; doi:10.1152/ajpcell.00184.2010.—The δ-subunit of the epithelial Na^+ channel (ENaC) is expressed in neurons of the human and monkey central nervous system and forms voltage-independent, amiloride-sensitive Na^+ channels when expressed in heterologous systems. It has been proposed that δ-ENaC could affect neuronal excitability and participate in the transduction of ischemic signals during hypoxia or inflammation. The regulation of δ-ENaC activity is poorly understood. ENaC channels in kidney epithelial cells are regulated by the serum- and glucocorticoid-induced kinase 1 (SGK1). Recently, a new isoform of this kinase (SGK1.1) has been described in the central nervous system. Here we show that δ-ENaC isoforms and SGK1.1 are coexpressed in pyramidal neurons of the human and monkey (*Macaca fascicularis*) cerebral cortex. Coexpression of δβγ-ENaC and SGK1.1 in *Xenopus* oocytes increases amiloride-sensitive current and channel plasma membrane abundance. The kinase also exerts its effect when δ-subunits are expressed alone, indicating that the process is not dependent on accessory subunits or the presence of PY motifs in the channel. Furthermore, SGK1.1 action depends on its enzymatic activity and binding to phosphatidylinositol(4,5)-bisphosphate. Physiological or pharmacological activation of phospholipase C abrogates SGK1.1 interaction with the plasma membrane and modulation of δ-ENaC. Our data support a physiological role for SGK1.1 in the regulation of δ-ENaC through a pathway that differs from the classical one and suggest that the kinase could serve as an integrator of different signaling pathways converging on the channel.

serum and glucocorticoid-induced kinase 1; voltage-independent Na^+ channel

THE EPITHELIAL Na^+ channel (ENaC) is a member of the ENaC/degenerin (DEG) family of ion channels (3). Its best known physiological role is to serve as rate-limiting step in transepithelial Na^+ reabsorption in tight epithelia such as the distal tubule of the kidney. Canonical ENaC channels are formed by three similar subunits, named α, β, and γ (10). Soon after the cloning of the channel, a fourth subunit, named δ, was identified in humans (41). Surprisingly, the δ-subunit has been found to be highly expressed outside epithelia, especially in the central nervous system (CNS), where it is exclusively neuronal, and in the pancreas (12). δ-ENaC is expressed as two splice isoforms with divergent NH_2 termini in human and primates (12, 43), but it is a pseudogene in rodents (GenBank accession no. NG_011905.1). It is able to form amiloride-sensitive, voltage-independent Na^+ channels when expressed alone or in combination with β- and γ-subunits (41). Its role in the CNS is uncertain, although its biophysical properties point toward several possibilities. First, being a voltage-independent, highly selective Na^+ channel, it could serve as a leak Na^+ conductance, contributing to the setting of resting membrane potential. In addition, δ-ENaC currents are enhanced by a drop in extracellular pH (pH_e) (19), suggesting that it could serve as a proton sensor and be involved in the transduction of ischemic signals that occur under conditions of tissue hypoxia or inflammation. In addition to its pathophysiological role, it is clear that a constitutively active Na^+ channel like ENaC has to be tightly regulated in nonepithelial cells to avoid cell death due to Na^+ loading and the loss of the electrochemical gradient in the membrane. Therefore, it is essential to uncover the molecular mechanisms involved in the control of δ-ENaC activity to advance our understanding of the role of this channel in neurons.

Whereas ENaC regulation by hormones and other stimuli has been studied extensively in epithelial cells expressing the canonical αβγ channel, very little is known about the regulation of channels formed by the δ-subunit. One of the major regulators of ENaC activity in kidney epithelial cells is the serum- and glucocorticoid-induced kinase 1 (SGK1), a serine-threonine kinase originally identified as a gene controlled by glucocorticoids (42) and changes in cell volume (40). SGK1 transcription is regulated by many different stimuli, including aldosterone (24). Its activation depends on the phosphoinositide 3 (PI3) kinase pathway (30) and probably represents a convergence point of different signaling pathways regulating ENaC (2, 31). SGK1 acts mainly by enhancing steady-state ENaC abundance in the plasma membrane (5). ENaC endocytosis is promoted by the activity of the ubiquitin ligase Nedd4–2, which interacts with ENaC subunits through a COOH-terminal PY motif (PPxY) (21, 36). SGK1 phosphorylates Nedd4–2 and disrupts its interaction with ENaC, stabilizing the channel in the membrane (11, 35).

Recently, a new splice isoform of SGK1, named SGK1.1, was characterized and found to be highly expressed in the mouse nervous system, where it downregulates acid-sensing ion channel 1 (ASIC1), another member of the ENaC/DEG

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family (7). SGK1.1 and SGK1 differ in their NH_2 terminus, which in turn determines a higher protein stability and increased plasma membrane binding for SGK1.1 (7, 33).

We hypothesized that SGK1.1 may be a regulator of δ-ENaC activity. Toward this end, we examined colocalization of SGK1.1 with δ-ENaC in human and monkey CNS, as well as the effect of the kinase on δ-ENaC activity in *Xenopus* oocytes. We demonstrate extensive colocalization of SGK1.1 and δ-ENaC isoforms in pyramidal neurons. SGK1.1 increases δ-ENaC current by increasing channel abundance in the plasma membrane. The effect does not require PY motifs and therefore appears to be independent of Nedd4–2. Furthermore, our data demonstrate that SGK1.1 effects on the channel can be abrogated by phospholipase C (PLC) activation, suggesting that the kinase plays a role as a converging point of signaling pathways regulating δ-ENaC channels.

MATERIALS AND METHODS

RT-PCR, DNA cloning, mutagenesis, and cRNA synthesis. The α, β, and γ ENaC subunits were amplified by RT-PCR from human lung RNA. The δ1- and δ2-subunits were obtained from the human bronchiolar epithelial H441 cell line RNA, which express both δ-isoforms (20). PCR products were purified and subcloned in pTNT vector (Promega, Mannheim, Germany) using restriction enzyme sites added to the oligonucleotides (*Eco*RI for α, γ, δ1, and δ2; *Not*I for β). Inserts were fully sequenced and compared with published sequences to ensure the absence of mutations. A human large-conductance Ca^{2+}-gated K^+ channel (BK) tagged with yellow fluorescent protein (YFP) has been previously described (14).

To generate fluorescently labeled δ1 and δ2, cDNAs were amplified by PCR with primers GAGAATTCGCCACCATGGCTGAGCACCGAAGCATGGAC (forward δ1), GACTGAATTCGCCACCATGGCTTTCCTCTCCAGGACG (forward δ2) and CAGAATTCGGGTGTCCAGAGTCTCAAGGGGCTG (reverse, common to both isoforms) using the pTNT expression vectors described above as templates. The products were subcloned in pEYFP-N1 (Clontech, Mountain View, CA) to produce an in-frame fusion of the YFP coding sequence. The δ_{YFP}-fusions were then subcloned in pTNT for expression in *Xenopus* oocytes.

Mouse SGK1.1 cloned in pcDNA3.1/V5-His-TOPO (Invitrogen) was a kind gift from Dr. Cecilia M. Canessa (Yale University, New Haven, CT). Point mutations in the SGK1.1 sequence were introduced with the Quickchange Lightning Site-directed Mutagenesis Kit (Agilent Technologies, Madrid, Spain) following the manufacturer's instructions. Silent mutations were introduced in each case for rapid screening of mutant clones (*Pst*I for K220A and *Fsp*I for K21N/K22N/R23G). Oligonucleotide sequences were as follows (mutant bases shown in lowercase): K220A, CTATGCAGTCgcAGTTcTgCAGAAGAAAGCCATCCTGAAGAAG (forward) and CTTCTTCAGGATGGCTTTCTTCTGcAgAACTgcGACTGCATAG (reverse); K21N/K22N/R23G, GCTCAGCGTTCCAATTTTTTAAcAAcgGGGTgCGcAGATGGATC (forward) and GATCCATCTgCGcACCCcgTTgTTAAAAAATTGGAACGCTGAGC (reverse). All mutations were confirmed by DNA sequencing. To generate fluorescently labeled SGK1.1, the cDNA was amplified by PCR with primers CGGAATTCGCCACCATGGTAAACAAAGACATGAATGG (forward) and GCGGATCCCGGAGGAAGGAATCCACAGGAGGTG (reverse) and then subcloned in pECFP-N1 (Clontech) to produce an in-frame fusion to the cyan fluorescent protein (CFP). SGK1.1-CFP was then subcloned in pGEMHE.

After linearization with the appropriate restriction enzymes, constructs were used as templates for in vitro cRNA synthesis using a commercial system (mMessage mMachine; Ambion, Austin, TX). cRNAs were purified by LiCl precipitation, resuspended in water, and quantified by absorption spectroscopy with a Nanodrop. cRNA quality was assessed by denaturing agarose gel electrophoresis.

In situ hybridization. The expression of ENaC-δ1, -δ2, and SGK1.1 was studied by in situ hybridization histochemistry in monkey (*Macaca fascicularis*) and human cerebral cortex. Monkey samples were provided by Dr. J. L. Lanciego (CIMA, University of Navarra, Pamplona, Spain). The experimental protocol was approved by the Ethical Committee of the University of Navarra (reference 001/006) and was in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) regarding the care and use of animals for experimental procedures. Human brains were provided by the Brain Bank of Navarra (Dr. T. Tuñón, Hospital de Navarra, Servicio Navarro de Salud, CIMA, Pamplona, Spain). They came from five patients (3 men and 2 women; average age 61.2 ± 5.3 yr) who died without history of drug abuse or neurological or psychiatric illness. Brains were removed after a postmortem period of 10.1 ± 2.4 h. In each case, the absence of degenerative or vascular disease was confirmed by pathological examination. Blocks containing the frontal and temporal cortices were briefly washed in 0.1 mol/l phosphate-buffered saline (PBS), pH 7.4, and immediately immersed in 4% paraformaldehyde in PBS for 72 h at 4°C. Four adult male monkeys (5–8 yr old, 3.5–4.8 kg) were administered an overdose of pentobarbital sodium and were transcardially perfused with heparinized ice-cold 0.9% saline followed by 3–4 liters of 4% paraformaldehyde in PBS. Brains were removed, cut into blocks, and immersed in fixation solution overnight. Human and monkey samples were cryoprotected by consecutive immersion in 10%, 20%, and 30% sucrose in PBS (24 h each), frozen, and cut into 40-μm-thick sections perpendicular to the long axis of the cortical gyri in human cortex and in the coronal axis in monkeys, with a freezing microtome.

In situ hybridization probes for δ1- and δ2-isoforms consisted of sense and antisense biotin-labeled 40-mer oligonucleotide probes and have been previously described (12). Sense oligonucleotides were used as control for nonspecific binding. A 511-bp fragment of human SGK1.1 (−295 to +216 relative to the start of the coding sequence) was amplified by PCR from human brain cortex cDNA using the following oligonucleotides: gagattggccgtatcccaccgtcc (forward) and gcatgttcacccaggcatgtttgac (reverse). This sequence does not overlap to the other known SGK1 isoforms (6, 7). The PCR product was purified and cloned in pCR4-TOPO (Invitrogen, Barcelona, Spain). Insert identity and orientation were verified by DNA sequencing. Sense and antisense digoxigenin (DIG)-labeled cRNA probes for in situ hybridization were made by in vitro transcription using T7 or T3 RNA polymerases and a commercially available kit (DIG RNA labeling kit, Roche, Barcelona, Spain). Labeling efficiency was determined by direct detection of the probes in a spot test.

SGK1.1 detection by single in situ hybridization labeling was performed as previously described (12, 15). Briefly, sections were prehybridized at 45°C for 2 h in hybridization solution (50% formamide, 5× SSC, and 40 μg/ml denatured salmon DNA). Probes were added to the hybridization mix at 400 ng/ml, and sections were incubated at 45°C for 16 h. Posthybridization washes included 2× SSC at 22°C for 10 min, 2× SSC at 55°C for 15 min, and 0.1× SSC at 55°C for 15 min. The slides were then equilibrated for 5 min in TN buffer (TNB, 100 mmol/l Tris·HCl and 150 mmol/l NaCl, pH 7.5) and incubated for 2 h at 22°C with alkaline-phosphatase-conjugated anti-DIG monoclonal antibody (1:2,500 final dilution in TN with 0.5% blocking reagent; Roche). After washes, the slides were equilibrated for 5 min in TNM buffer (100 mmol/l Tris·HCl, 100 mmol/l NaCl, and 50 mmol/l $MgCl_2$, pH 9.5) and incubated in substrate solution (Nitro-Blue tetrazolium chloride and 5-bromo-4-chloro-3′-indolyphosphate *p*-toluidine salt in TNM buffer; Roche). Staining was stopped in TE (10 mmol/l Tris·HCl and 1 mmol/l EDTA, pH 8.0), and the slides were dehydrated and mounted in Entellan (Merck, Darmstadt, Germany).

For double in situ hybridization labeling, biotin- and DIG-labeled probes were simultaneously added to the hybridization mix. The combination of probes that gave optimal results was biotin-δ1 or -δ2 and DIG-SGK1.1. The fluorescent visualization of the biotin-labeled

probe was carried out first. After the final wash in 0.1× SSC, sections were equilibrated in TNB for 30 min and were then incubated with streptavidin-horseradish peroxidase (1:150, PerkinElmer, Madrid, Spain) in TNB buffer for 30 min at room temperature. After several washes with TNT (Tris-NaCl-Tween 20) buffer, the sections were incubated for 10 min in biotinyl tyramide (1:75 in amplification diluent; PerkinElmer). Fluorescence was developed using Cy2-conjugated streptavidin (GE Healthcare, Madrid, Spain). The second transcript was detected with a DIG-labeled riboprobe that was visualized after the biotin-labeled probe. The sections were briefly rinsed with TNB and incubated for 90 min at room temperature with a sheep anti-DIG antibody conjugated to alkaline phosphatase (1:500; Roche). After several rinses in TNT buffer, sections were washed twice for 10 min with TNM buffer at room temperature and transcripts were visualized using the HNPP fluorescence detection kit (Roche). To eliminate autofluorescence arising from lipofusin deposition, sections were incubated in 5 mM $CuSO_4$ and 50 mM ammonium acetate pH 5.0 for 10 min. Thereafter, they were mounted on glass slides, air-dried at room temperature in the darkness, rapidly dehydrated in toluene, and coverslipped with DPX (BDH Chemicals, Barcelona, Spain). Images were obtained under a Leica DMR photomicroscope (Leica Microsystems, Barcelona, Spain) or a FluoView 1000 confocal microscope (Olympus, Barcelona, Spain) and compiled using Adobe Illustrator software (Adobe Systems, San Jose, CA).

ENaC heterologous expression in Xenopus laevis oocytes and electrophysiology. All procedures involving *Xenopus laevis* were approved by the University of La Laguna Research Ethics Committee in agreement with local and national legislation. Adult females were anesthetized by immersion in fresh water containing 1 g/l tricaine (Sigma, St. Louis, MO) and buffered to pH 7.2–7.3 with HEPES. Oocytes were harvested by partial ovariectomy and collagenase IA (Sigma) dispersion. Stage V to VI oocytes were selected and microinjected with 2–2.5 ng of full-length human ENaC subunits (δ1 or δ2 alone or in combination with β and γ) or BK cRNAs. SGK1.1 or its mutant cRNAs were coinjected at a ratio of 5:1 (kinase:channel). Oocytes were then incubated for 1–2 days at 16°C in oocyte Ringer medium (in mmol/l: 82.5 NaCl, 2 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 1 Na_2HPO_4, and 10 HEPES, pH 7.5) supplemented with 100 μmol/l amiloride (Sigma). In some cases, amiloride was not used and instead Na^+ was largely replaced by *N*-methyl-D-glucamine in the incubation medium, which consisted of (in mM) 10 NaCl, 80 NMDG, 1 KCl, 2 CaCl2, 5 HEPES, 2.5 Na pyruvate, 0.06 penicillin, and 0.02 streptomycin, pH 7.4. Oocyte whole cell currents were recorded using a two-electrode voltage-clamp (TEVC) system with a OC-725C amplifier (Warner Instruments, Hamden, CT) as described previously (12, 29). The bath solution contained (in mmol/l) 150 Na^+ gluconate, 1.8 $CaCl_2$, 2 $MgCl_2$, 4 KCl, 5 $BaCl_2$, and 5 HEPES, pH 7.2. ENaC-specific currents were calculated as the difference before and after the addition of 100 μmol/l amiloride to the bath. Current-voltage curves were generated by increasing voltage from −70 to +40 mV in sequential 10-mV steps of 100-ms duration each. In the case of BK currents, recording was performed in oocyte Ringer medium by application of voltage pulses to +100 mV from a holding potential of −70 mV. Currents were recorded at 1 kHz, except in experiments for Fig. 7, for which they were recorded at 100 Hz. Stimulation and data acquisition were controlled using pClamp 10.0 software (Axon Instruments, Sunnyvale, CA) running on a PC computer. Data analysis was performed with the programs Clampfit (Axon), Prism 5.0b (GraphPad Software, San Diego, CA), and Igor-Pro (WaveMetrics, Lake Oswego, OR).

Protein detection by Western blot and confocal microscopy. Oocyte protein extracts were prepared in lysis buffer containing 50 mmol/l Tris·HCl, pH 7.5, 5 mmol/l EDTA, 150 mmol/l NaCl, 1% Triton X-100, and a protease inhibitor cocktail (Roche). After the lysates were cleared by centrifugation, protein concentration was measured with the bicinchoninic acid procedure (Sigma). Equal amounts of protein were resolved by SDS-PAGE and transferred to Immobilon P membranes (Millipore, Madrid, Spain). Membranes were blocked with 5% dry milk, and YFP- or CFP-tagged protein expression was detected with anti-green fluorescent protein monoclonal antibody (Clontech) followed by incubation with goat anti-mouse secondary antibody conjugated to horseradish peroxidase (GE Healthcare). Chemiluminescence was developed with Immun-Star WesternC kit (Bio-Rad, Hercules, CA), and signals were detected with a Versadoc 4000 MP imaging system (Bio-Rad). Cell surface expression of YFP-tagged ENaC δ1- or δ2-subunits or CFP-tagged SGK1.1 in living oocytes was detected using a laser-scanning confocal microscope (Olympus FluoView 1000; Olympus) as described previously (12). For time course recordings, images were taken every 10 s. Background fluorescence was assessed by imaging noninjected oocytes.

Statistical analysis. Statistical analysis of electrophysiological and fluorescence recordings was done using Prism 5.0b software (GraphPad Software) to apply nonparametric two-tailed Mann-Whitney test or Wilcoxon signed-rank tests to the data. When more than two groups were compared, a nonparametric ANOVA Kruskal-Wallis test was used, followed by a Dunn's multiple-comparison test.

RESULTS

ENaC δ-subunit and SGK1.1 are coexpressed in pyramidal neurons of the human and monkey brain cortex. SGK1.1 mRNA and protein are expressed in the CNS, although its localization in specific cell types has not been described (7). To test whether SGK1.1 colocalizes with ENaC δ-subunit isoforms, we generated a DIG-labeled cRNA probe specific for SGK1.1 and performed in situ hybridization in sections obtained from human and monkey (*M. fascicularis*) cerebral cortex (Fig. 1). We observed staining in many neurons with pyramidal morphology through layers II to VI and the underlying white matter of the frontal and temporal cortices (Fig. 1, *A–C*). Interestingly, we consistently observed lower expression levels of SGK1.1 in layer IV. As a whole, this expression pattern resembles that of δ1- and δ2-ENaC (Fig. 1, *D* and *E*), previously described by our group (12), suggesting that δ-ENaC and SGK1.1 could be coexpressed in the same neurons. We further investigated this hypothesis by performing double fluorescent in situ hybridization with a DIG-labeled probe specific for SGK1.1 and biotin-labeled oligonucleotides specific for δ1- or δ2-ENaC. Our results show colocalization of δ-ENaC isoforms and SGK1.1 mRNAs in 91% of monkey (Fig. 1, *F–H*) and human (Fig. 1, *I–N*) pyramidal cells. Less than 10% of pyramidal cells, most of them small in size lying in layer IV and in the deep region of layer III, express δ-ENaC isoforms but not SGK1.1. SGK1.1-positive and δ-ENaC-negative cells were not detected. These results suggest that SGK1.1 could participate in the regulation of δ-ENaC in neurons.

SGK1.1 increases ENaC-δβγ activity in Xenopus oocytes. To test whether SGK1.1 modulates ENaC-δ channels, we used heterologous expression in *Xenopus* oocytes, where ENaC-δ subunits form functional channels with ENaC-β and -γ (12, 41). Channel activity was assessed as amiloride-sensitive membrane currents using TEVC. When SGK1.1 was coexpressed with δ1βγ channels, we observed a twofold increase in amiloride-sensitive current levels at all voltages tested (Fig. 2, *A–C*). To test whether this effect is dependent on the kinase activity of SGK1.1, we generated a mutant that substitutes a lysine residue in the ATP-binding cassette of the protein (K220, equivalent to K127 in human SGK1) abolishing kinase

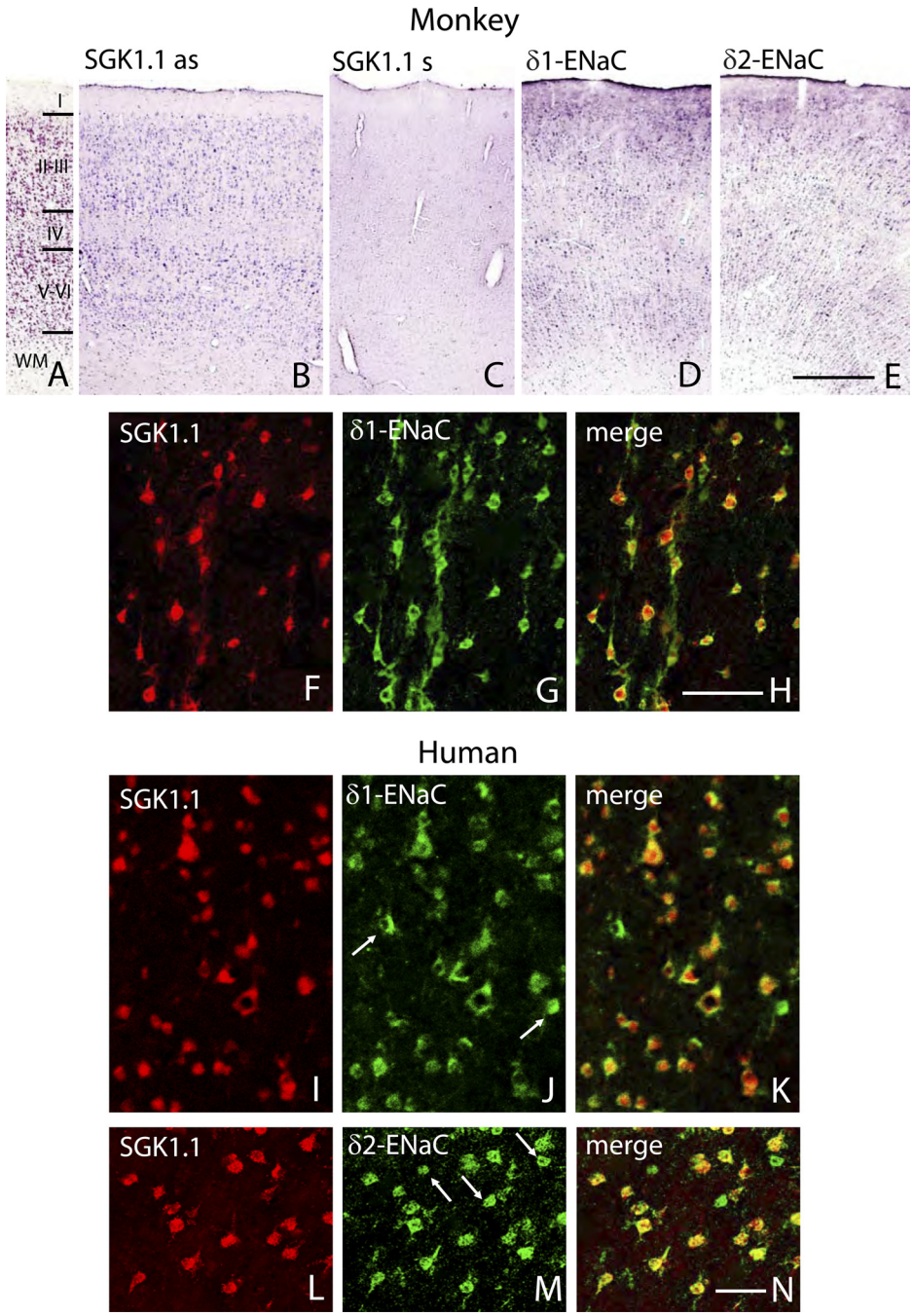

Fig. 1. Serum- and glucocorticoid-induced kinase 1 isoform SGK1.1 and δ-subunit isoform of epithelial Na^+ channel (ENaC) are coexpressed in pyramidal neurons of monkey and human cerebral cortex. *A*: Nissl staining showing the layering in the monkey temporal cortex. WM, white matter. *B–E*: single colorimetric in situ hybridization for SGK1.1 antisense (as, *B*) and sense (s, *C*) riboprobes, and for δ1 (*D*) and δ2 (*E*) ENaC isoforms in the monkey temporal cortex. *F–H*: double fluorescent in situ hybridization for SGK1.1 and δ1-ENaC in layer II of the monkey temporal cortex. *I–K*: double fluorescent in situ hybridization for SGK1.1 and δ1-ENaC in layers II-III of the human frontal cortex. *L–N*: double fluorescent in situ hybridization for SGK1.1 and δ2-ENaC in layer III of the human temporal cortex. Arrows in *J* and *M* indicate neurons expressing δ-ENaC isoforms but not SGK1.1. Bar in *E* (for *A–E*), 750 μm; in *H* (for *F–H*), 100 μm; in *N* (for *I–N*), 50 μm.

activity (30). SGK1.1-K220A did not produce significant changes in channel activity (Fig. 2, *B* and *C*). The SGK1.1-mediated increase in current was also observed with channels incorporating the ENaC δ2-isoform (Fig. 2*C*). Since ENaC channels are constitutively active and despite keeping oocytes in 100 μmol/l amiloride, they were overloaded with Na^+ and thus reversal potential was shifted to more negative values. This shift was larger when the SGK1.1 was coexpressed (Fig. 2*B*). To study whether the increased current was due to an increase in the abundance of channels at the membrane, we used δ-subunits fluorescently labeled by the addition of YFP to the COOH terminus. Oocytes expressing ENaC $\delta 1_{YFP}\beta\gamma$ channels and SGK1.1 showed a 2.5-fold increase in cell surface expression of the labeled subunit compared with those expressing either the channels alone or the channel and the inactive kinase K220A (Fig. 2, *D* and *F*). Western blot analysis of the same oocytes showed that SGK1.1 effects cannot be explained by changes in δ1 total protein abundance (Fig. 2*E* and quantified in Fig. 2*F*), indicating that the increase in plasma membrane expression is due to a change in channel trafficking. We obtained similar results with the ENaC δ2βγ-subunit combination (data not shown). Taken together, these results indicate that SGK1.1 increases expression of ENaC-δβγ channels at the plasma membrane, thus increasing Na^+ current, through a mechanism that depends on the kinase activity of SGK1.1.

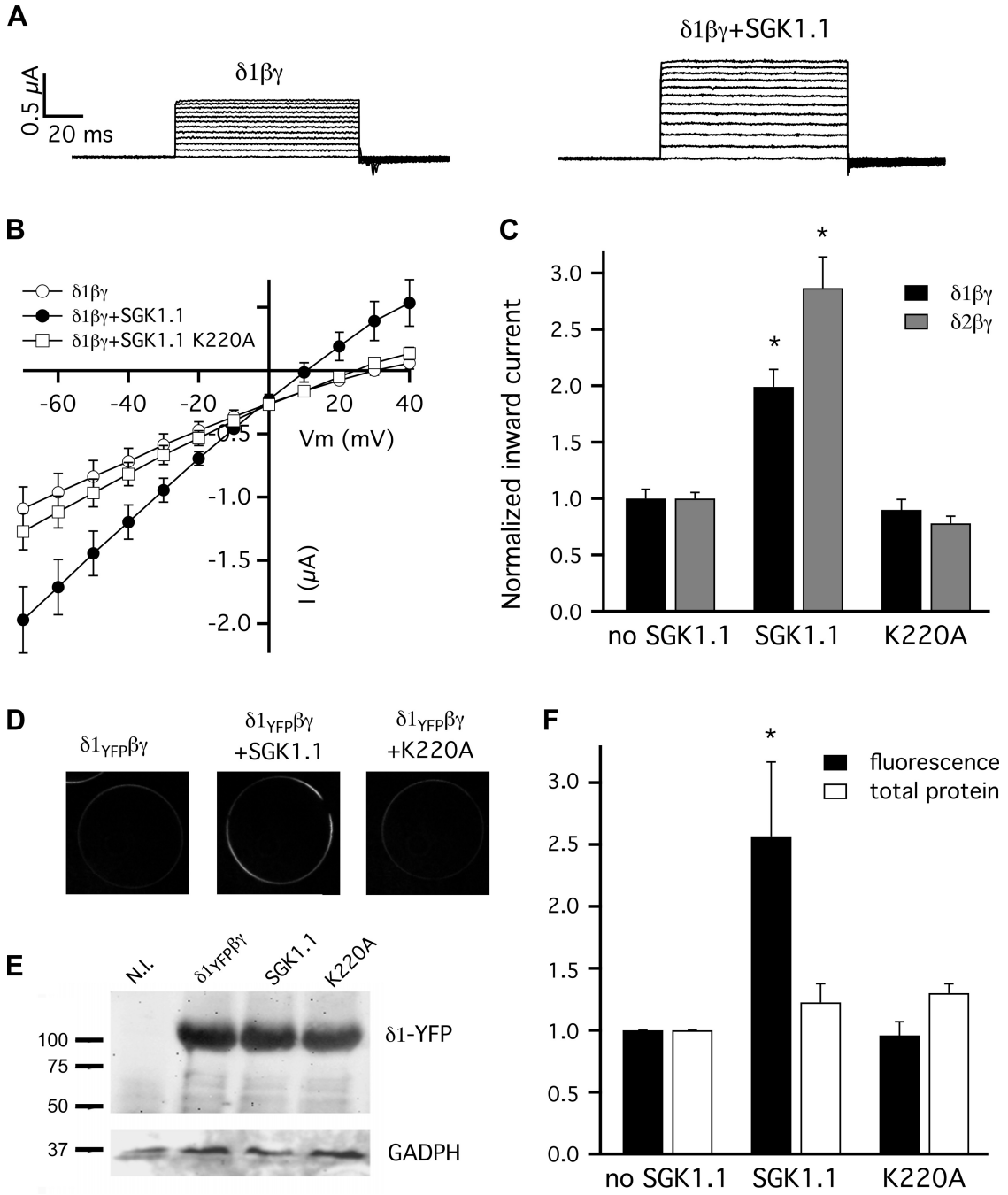

Fig. 2. SGK1.1 increases δβγ-ENaC currents by increasing channel plasma membrane abundance. *A*: currents elicited by coinjection of ENaC-δ1βγ with or without SGK1.1 cRNAs. Shown are representative amiloride-sensitive currents obtained by increasing voltage from −70 mV to +40 mV in sequential 10-mV steps. *B*: representative current-voltage (*I-V*) curves obtained from one batch of oocytes injected with δ1βγ alone, in combination with SGK1.1, or with SGK1.1-K220A mutant. Data points represent current average ± SE ($n = 6$). V_m, membrane potential. *C*: amiloride-sensitive current magnitude averages at a holding potential of −60 mV obtained from 3–4 batches of oocytes coinjected with δ1βγ (black bars) or δ2βγ-subunits (gray bars), with or without SGK1.1, or the K220A mutant of the kinase. Error bars represent SE ($n > 60$ for each condition). *$P < 0.05$, Kruskal-Wallis nonparametric test followed by a Dunn's multicomparison test. *D*: representative confocal images showing cell surface expression of fluorescently labeled δ-ENaC in *Xenopus* oocytes without SGK1.1, with SGK1.1, or with the mutant SGK1.1-K220A. YFP, yellow fluorescent protein. *E*: Western blot analysis of δ_{YFP}-expression in *Xenopus* oocytes expressing $\delta1_{YFP}\beta\gamma$ alone or in combination with SGK1.1 or SGK1.1-K220A. Molecular mass marker migration is shown to the *left* (values are in kDa). NI, noninjected oocytes. *F*: black bars represent a quantification of average fluorescence intensity monitored in oocytes expressing $\delta1_{YFP}\beta\gamma$-ENaC without SGK1.1 ($n = 15$), with SGK1.1 ($n = 8$), and with the mutant SGK1.1 K220A ($n = 10$). White bars represent average total protein abundance quantified by Western blot analysis of four independent batches of oocytes. Error bars represent SE. *$P < 0.05$, Kruskal-Wallis nonparametric test followed by a Dunn's multicomparison test.

SGK1.1 increases the activity of ENaC-δ expressed alone in Xenopus oocytes. Since the δ-subunit of ENaC lacks a PY motif in the COOH terminus (Fig. 3*A*), we checked whether the presence of accessory β- and γ-subunits is required for the effect of SGK1.1 on channel activity. Expression of the δ-subunit alone produced amiloride-sensitive currents in *Xenopus* oocytes (Fig. 3*B*), although at a much lower level than that elicited by the combination δβγ, consistently with previous

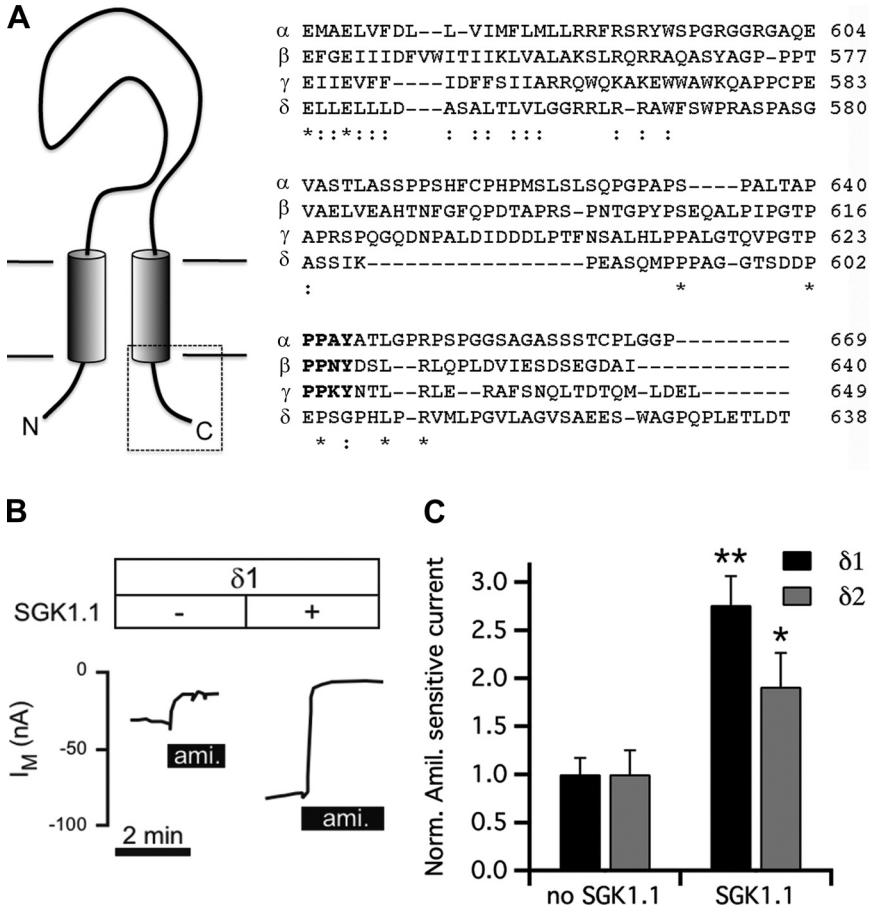

Fig. 3. SGK1.1 upregulates channels formed by the δ-subunit in a PY motif-independent fashion. *A*: schematic representation of ENaC subunit topology and COOH-terminal domain (box) sequence alignment of the human α-, β-, γ-, and δ-subunits. Sequence analysis was performed with Kalign 2.0 (European Bioinformatics Institute) (26). Numbering of the δ-subunit sequence refers to the δ1-isoform. PY motifs (PPxY) in α, β, and γ are shown in bold. *, identical residues; :, conserved substitutions. *B*: representative current traces of oocytes injected with δ1 alone or in combination with SGK1.1 and held at −60 mV in the absence or presence of 100 μM amiloride (Ami). *C*: normalized amiloride (Norm Amil)-sensitive current magnitude averages at a holding potential of −60 mV obtained from 3 batches of oocytes injected with δ1- (black bars) or δ2-subunits (gray bars), with or without SGK1.1; $n > 10$ for each condition. Error bars represent SE. $^*P < 0.05$; $^{**}P < 0.001$; two-tailed Mann-Whitney test.

observations (17, 41). Independent of the basal level of current, coexpression of SGK1.1 increased δ1- or δ2-ENaC currents by approximately 2.0- to 2.5-fold (Fig. 3, *B* and *C*), indicating that the presence of PY motif-containing β- and γ-subunits is not required for the regulation of δ-ENaC by the kinase.

SGK1.1 effect on δ-ENaC does not reflect a general change in the trafficking of membrane proteins. To ensure that the increase in δ-ENaC membrane expression is not a consequence of a general effect of SGK1.1 on cellular membrane trafficking, we investigated the effect of SGK1.1 on other ion channel currents in the oocyte. Coexpression of the kinase with a human BK channel tagged with YFP did not induce any variation in K^+ current or membrane expression levels of the channel (Fig. 4, *A* and *B*). We also tested whether SGK1.1 produced any change in oocytes endogenous currents, and no significant variation was observed (Fig. 4*C*). These results are further supported by other authors' findings showing that SGK1.1 does not affect endogenous voltage-activated Na^+ currents in neurons (7).

Basic residues in the NH_2-terminal region of SGK1.1 are needed for ENaC-δβγ regulation. In other cell types, SGK1.1 has been shown to reside at the plasma membrane by binding to phosphatidylinositol(4,5)-bisphosphate [PtdIns(4,5)P_2] (7). When the fluorescently labeled SGK1.1-CFP was expressed in *Xenopus* oocytes, we observed clear plasma membrane localization (Fig. 5*A*), demonstrating that this process is conserved in the oocyte expression system. PtdIns(4,5)P_2 binding by SGK1.1 depends on an NH_2-terminal polybasic motif including residues K21, K22, and R23 (7). In *Xenopus* oocytes, this also seems to be the case, because mutating those residues for neutral ones (SGK1.1-K21N/K22N/R23G) reduced membrane fluorescence to levels that are only slightly above those of noninjected oocytes (Fig. 5, *A* and *B*). This difference in cell surface expression is not due to diminished SGK1.1 protein expression, as demonstrated by Western blot analysis of the same oocytes (Fig. 5*C*). Therefore, SGK1.1 follows the same pattern of subcellular localization in *Xenopus* oocytes and in mammalian cells.

Coexpression of the SGK1.1 K21N/K22N/R23G mutant with ENaC-δ1βγ channels did not significantly increase the amiloride-sensitive current as opposed to the effect of the wild-type kinase (Fig. 5, *D* and *E*). Thus, these three residues are not only needed for the kinase to be bound to the membrane, but also to regulate δ1βγ function. The same result was obtained with δ2βγ channels (data not shown).

Activation of PLC leads to SGK1.1 removal from the membrane and abrogates its effects on δβγ channel activity. Since PLC activation transiently reduces PtdIns(4,5)P_2 levels at the plasma membrane, we asked whether the PLC pathway could modulate SGK1.1 effects on δ-ENaC currents. The effect of pharmacological activation of PLC with 3M3FBS (18) on SGK1.1-CFP plasma membrane localization was monitored in living oocytes using confocal microscopy. Within 20 s after addition of the activator, we observed a rapid decrease in fluorescence, which peaked at 25% of the baseline, indicating that SGK1.1 was being retrieved from the membrane (Fig. 6, *A*

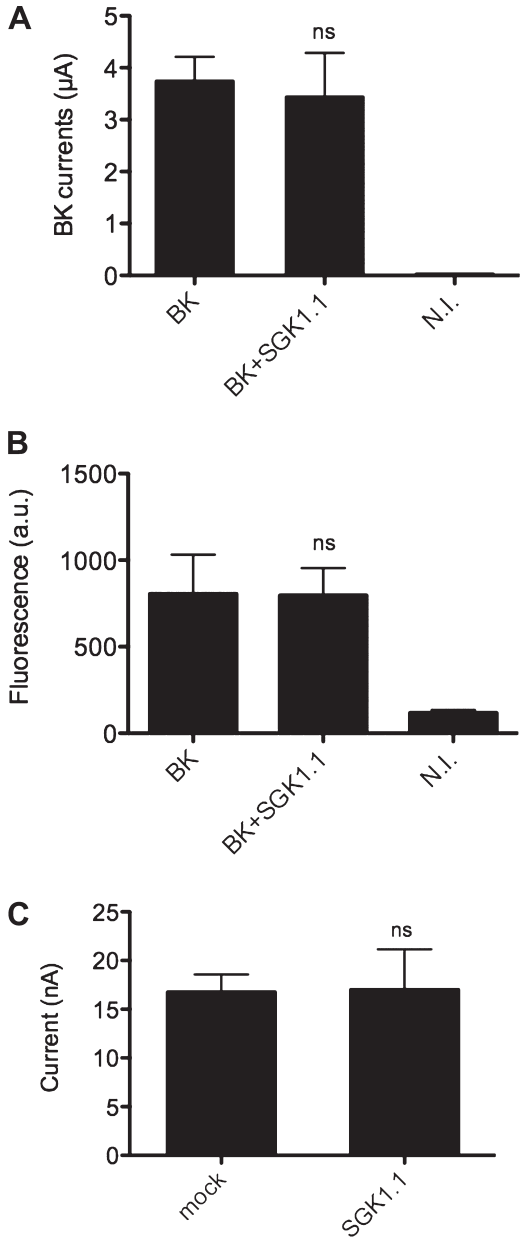

Fig. 4. SGK1.1 does not increase the expression of other ion channels at the plasma membrane. *A*: large-conductance Ca^{2+}-gated K^+ (BK) current magnitude averages at test voltage pulses (+100 mV from a holding of −70 mV) obtained from oocytes expressing BK-YFP channels alone ($n = 10$) or in combination with SGK1.1 ($n = 10$). AU, arbitrary units. *B*: average fluorescence intensity monitored by confocal microscopy in the same oocytes used for recording BK-YFP channel activity in *A*. *C*: endogenous current magnitude averages at a holding potential of 0 mV obtained from oocytes injected with H_2O, with or without SGK1.1 cRNA ($n = 12$ for each condition). Error bars represent SE. NS, not significant (two-tailed Mann-Whitney test).

and *B*). This same effect was observed when the oocytes were preincubated with 3M3FBS for 1–2 min, although the average decrease in this case was lower (60% of the baseline), probably because PLC activation had been partially reversed [Fig. 6*C* (18)]. The effect of 3M3FBS was not observed in oocytes expressing the SGK1.1-CFP K21N/K22N/R23G mutant, which is not bound to the membrane. In this case, addition of the PLC activator did not further reduce the remaining membrane fluorescence, indicating that this result is due to PLC-mediated PtdIns(4,5)P_2 hydrolysis (Fig. 6*C*).

Since binding of SGK1.1 to the membrane is necessary to regulate δβγ channels, we speculated that PLC-mediated removal of SGK1.1 from the membrane would consequently lead to reduced δβγ current levels. To test this hypothesis, we measured amiloride-sensitive inward currents before and after 3M3FBS incubation of oocytes injected with $\delta_1\beta\gamma$-ENaC, with or without SGK1.1. Our results show no significant changes in $\delta_1\beta\gamma$-ENaC currents after 30 min of incubation with the PLC activator when SGK1.1 is not coinjected (Fig. 6*D*, *left*). However, we observed a significant reduction of amiloride-sensitive inward current in oocytes coexpressing SGK1.1 (Fig. 6*D*, *right*), suggesting that PLC activation at least partially abrogates the kinase effect on δβγ-ENaC.

One of the most common physiological situations in which activation of PLC is involved is the activation of G protein-coupled receptors (GPCR) at the membrane. *Xenopus* oocytes endogenously express lysophosphatidic acid (LPA) receptors, which are coupled to various signaling cascades that involve PLC activation (38). To test whether physiological activation of PLC through the activation of an endogenous GPCR could modulate SGK1.1 subcellular localization, we monitored the membrane fluorescence of oocytes expressing SGK1.1-CFP before and after addition of LPA to the medium. As shown in Fig. 7*A*, we observed a significant and sustained reduction of SGK1.1 membrane levels to an average 60% of its initial value (Fig. 7*A*).

We then tested whether the removal of SGK1.1 from the membrane, now as a result of the physiological activation of GPCR, would consequently lead to reduced δβγ current levels. We measured amiloride-sensitive inward currents before and after LPA addition in oocytes held at −70 mV. Initial ENaC activity was calculated by the addition of 100 μM amiloride. LPA was added in the presence of amiloride to avoid Na^+ loading of the oocytes. Amiloride was washed at the end of the experiment to calculate the remaining ENaC activity. Representative traces from oocytes injected with δ1βγ alone or in combination with SGK1.1 are shown in Fig. 7*B*. Soon after addition of LPA, a transient inward current corresponding to Ca^{2+}-dependent activation of Cl^- channels (13) was observed. Our results show that LPA did not produce a statistically significant change in the amiloride-sensitive inward current in oocytes expressing only δ1βγ channels (Fig. 7*C*), although a tendency toward increased current was observed. On the contrary, when SGK1.1 was coexpressed with δ1βγ channels, LPA produced a significant reduction of the amiloride-sensitive current (Fig. 7*C*), averaging a 40% decrease. As a control to test the specificity of the LPA effect on δβγ-ENaC, we measured the amount of Ca^{2+}-induced Cl^- current from the recordings, observing no significant change with or without SGK1.1 (Fig. 7*D*). These data demonstrate that activation of PLC signaling through an endogenous GPCR can be linked to the modulation of δβγ channels through SGK1.1, proposing a mechanism of ENaC-δ channels regulation in neurons.

DISCUSSION

In this work we have presented evidence supporting a role for SGK1.1 in the control of neuronal Na^+ channels formed by the δ-subunit of ENaC. Both proteins colocalize in pyramidal

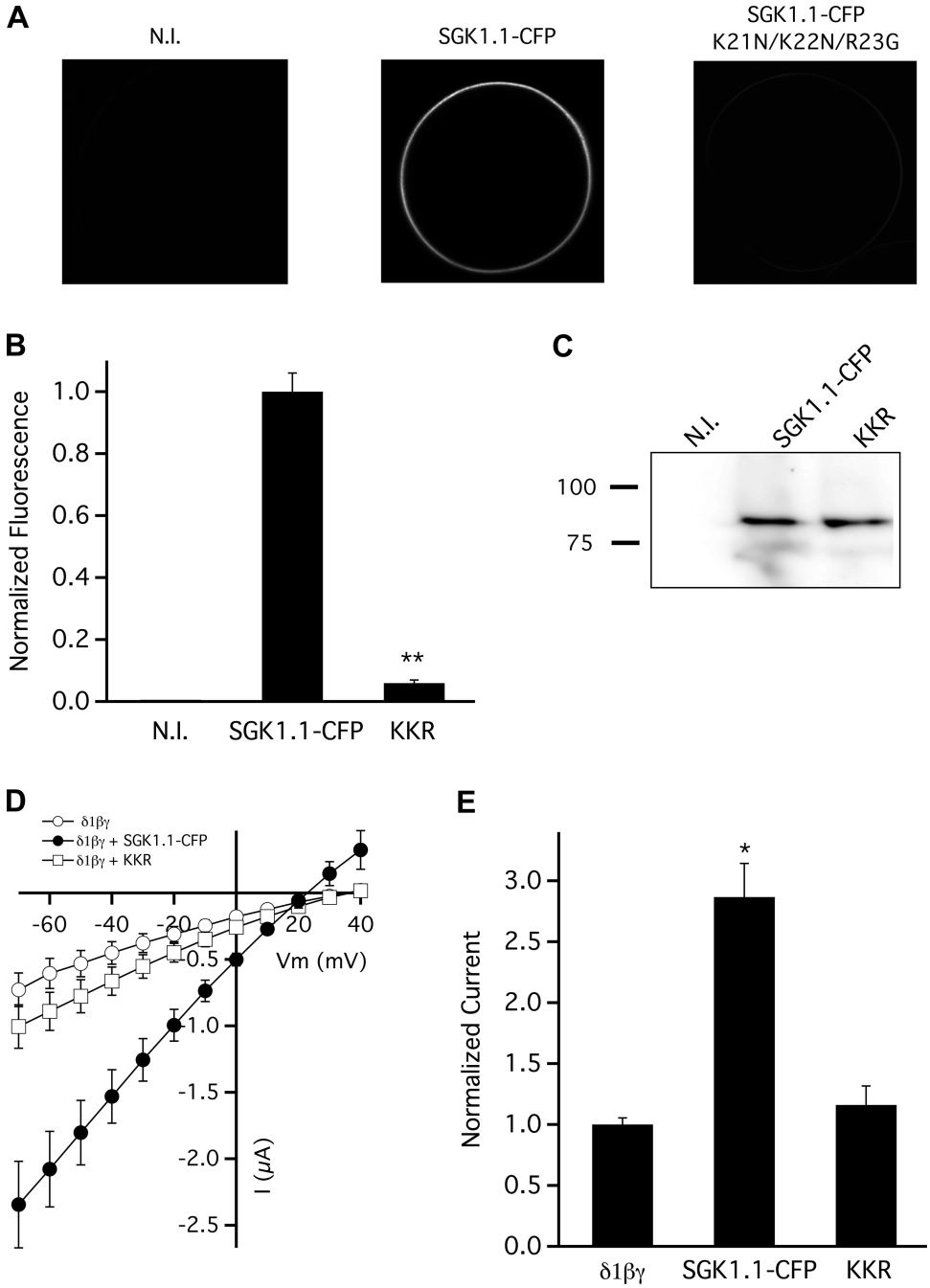

Fig. 5. Basic residues in the SGK1.1 NH_2-terminal domain are needed for membrane localization and δβγ channel regulation. *A*: representative confocal images showing cell surface expression of SGK1.1-cyan fluorescent protein (CFP) and SGK1.1-K21N/K22N/R23G (KKR) mutant in *Xenopus* oocytes. *B*: quantitative representation of average fluorescence intensity monitored in noninjected oocytes and oocytes expressing SGK1.1-CFP and KKR mutants. Error bars represent SE. $^{**}P < 0.001$ ($n = 20$), two-tailed Mann-Whitney test. *C*: SGK1.1 protein expression analysis by Western blot from noninjected oocytes, or oocytes injected with SGK1.1-CFP or KKR mutant. Migration of 100 kDa and 75 kDa molecular mass standards is shown to the *left*. *D*: representative *I-V* curves obtained from one batch of oocytes injected with δ1βγ alone, with SGK1.1-CFP, or with SGK1.1-CFP-KKR. Data points represent current average ± SE ($n = 15$). *E*: average amiloride-sensitive current magnitudes at a holding potential of −60 mV obtained from 3–4 batches of oocytes injected with δ1βγ, δ1βγ + SGK1.1-CFP, and δ1βγ + KKR. Error bars represent SE ($n > 60$ for every condition). $^{*}P < 0.05$, Kruskal-Wallis nonparametric test followed by a Dunn's multicomparison test.

neurons of the monkey and human brain cortex. When coexpressed in a heterologous expression system, SGK1.1 enhances the activity of channels formed by δβγ-subunits or by the δ-subunit alone, regardless of the isoform of δ present in the heteromer. The effect of SGK1.1 depends on its enzymatic activity and binding to membrane phospholipids. Pharmacological or physiological activation of PLC abrogates SGK1.1 effect on δ-ENaC, suggesting that the kinase provides a molecular link between PLC activation and the control of δ-ENaC activity.

Cellular localization of SGK1.1 in the human and monkey cerebral cortex. Initial characterization of SGK1.1 expression clearly showed that its mRNA is highly expressed in the mouse and human CNS, although there may be species-specific differences regarding expression in other tissues (7, 33). Moreover, it was shown that because of increased protein stability, SGK1.1 is the predominant isoform expressed in the mouse brain (7). However, the precise cellular localization pattern of SGK1.1 in the brain has not been described. In this work we present evidence supporting a high level of expression of this kinase in pyramidal neurons of the human and monkey cerebral cortex, except in layer IV, where expression is clearly diminished. The use of double fluorescent in situ hybridization allowed us to demonstrate a high degree of colocalization between SGK1.1 and δ-ENaC isoforms. We did not detect pyramidal neurons expressing SGK1.1 but not δ-ENaC. In our

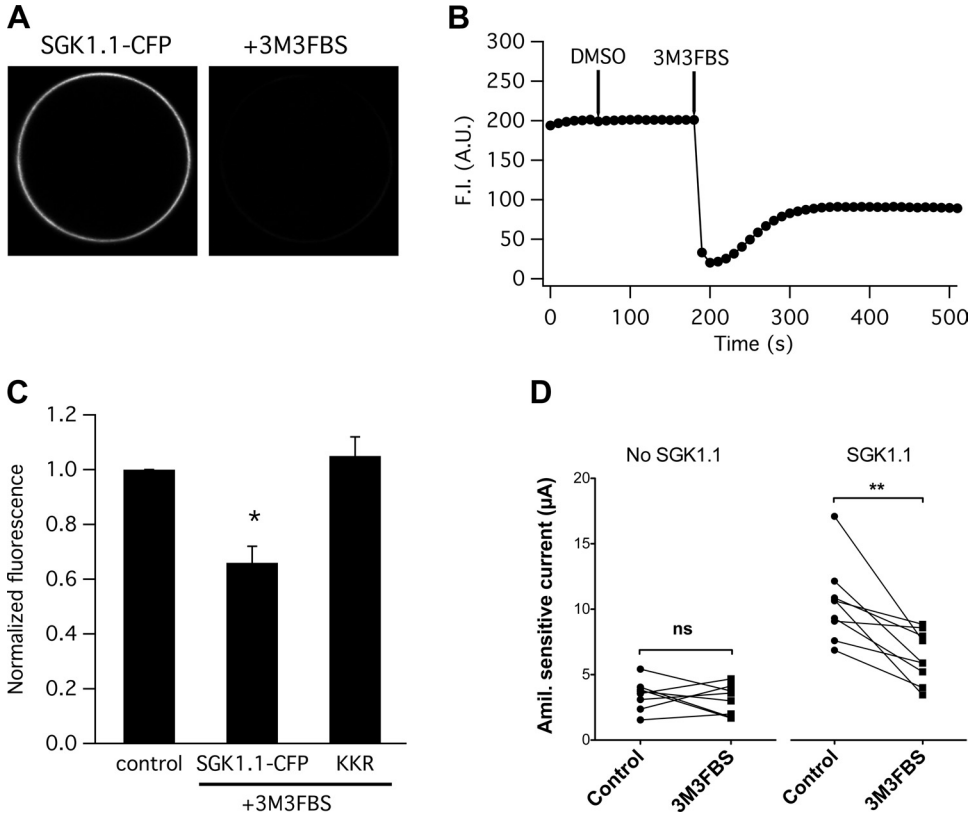

Fig. 6. Activation of phospholipase C (PLC) with 3M3FBS removes SGK1.1-CFP from the oocyte membrane and partially reverses SGK1.1 effect on δβγ channels. *A*: representative confocal microscope images of oocytes injected with SGK1.1-CFP in the absence or presence of 3M3FBS. *B*: representative time course recording of membrane fluorescence intensity (FI) of SGK1.1-CFP-injected oocytes. Addition of DMSO and 3M3FBS is shown with vertical bars above the trace. Images were taken every 10 s. *C*: quantitative representation of normalized fluorescence intensity obtained from oocytes expressing SGK1.1-CFP or SGK1.1-CFP-KKR before (control) and after 2–3 min incubation with 3M3FBS. Each treatment was normalized to its control. Error bars represent SE ($n = 10$). *$P < 0.05$, Kruskal-Wallis nonparametric test followed by a Dunn's multicomparison test. *D*: quantitative representation of amiloride-sensitive currents compared before and after incubation of oocytes with 3M3FBS (10 μmol/l in 47 nl, 30 min), expressing δ1βγ alone ($n = 6$) or δ1βγ with SGK1.1 ($n = 8$). **$P < 0.01$, Wilcoxon signed-rank test.

previously published work (12), we performed double staining experiments that excluded the expression of δ-ENaC in non-pyramidal neurons or glial cells in the human or monkey cerebral cortex. Therefore, we can conclude that SGK1.1 expression appears to be restricted to cortical pyramidal neurons as well. Expression in other cell types was not apparent by in situ hybridization, although it cannot be excluded that they express low levels of mRNA that fall under the detection threshold of our technique. Most importantly, coexpression of δ-ENaC and SGK1.1 indicates that the functional relationship found in oocytes could be relevant in pyramidal neuron physiology.

Mechanisms of δ-ENaC regulation by SGK1.1. It is well established that the ubiquitous kinase SGK1 upregulates the canonical αβγ-ENaC channel and participates in the regulation of transepithelial Na^+ transport (24). This effect is mainly due to an increased abundance of the channel at the plasma membrane (5), although it has also been demonstrated that SGK1 produces a change in ENaC open probability (P_o) (4, 39). This second mechanism could be indirect, reflecting different rates of endocytosis of high P_o vs. low P_o ENaC (34). Our results show that SGK1.1 also affects δβγ-ENaC trafficking, stabilizing the channel at the plasma membrane, which in turn can account for the increase in whole cell current. The effects of SGK1 on αβγ-trafficking are mediated, at least in part, by phosphorylation and subsequent inactivation of Nedd4–2 (11, 35), a ubiquitin ligase that binds ENaC subunit COOH-terminal PY motifs and ubiquitinates the channel, promoting its endocytosis. The δ-subunit lacks PY motifs and would therefore depend on the presence of β- and/or γ-subunits for this mechanism to take place. However, our results contradict this hypothesis, since SGK1.1 upregulates ENaC channels formed only by δ-subunits, indicating that the kinase acts through a PY motif-independent pathway. Alternative mechanisms of SGK1 action have been observed and include a Rab4-dependent facilitation of AMPA receptor recycling to the membrane in cultured cortical neurons (27) and an increased insertion of the kainate receptor GluR6 into the membrane (37). Moreover, the neuronal SGK1.1 has been shown to downregulate another member of the ENaC/DEG family, ASIC1, by decreasing its abundance in the plasma membrane (7). Taken together, the available information clearly indicates that the modulation of membrane protein expression by SGK1.1 is specific to the vesicular cargo and is not a general effect on cellular membrane trafficking. The precise mechanisms underlying the specific effect of SGK1.1 on different neuronal channels warrant further investigation.

Physiological roles of SGK1.1 and the regulation of δ-ENaC in the nervous system. Whereas there is little information available regarding the roles of SGK1.1 in the nervous system, SGK1 has been implicated in a wide variety of physiological, pathological, and pharmacological processes in the brain (25). Some of SGK1 effects are mediated by modulation of ion channel or transporter activity. For instance, SGK1 regulates several glutamate transporters, voltage-dependent K^+ channels (25), and AMPA and kainate glutamate receptors (27, 37), indicating that the kinase could be involved in the modulation of synaptic transmission, plasticity, and neuronal membrane potential. It is important to note that most of the studies addressing the effects of SGK1 on neuronal ion channels or transporters have been performed in heterologous expression systems. Given that SGK1.1 is the predominant isoform in the

brain under physiological conditions (7) and the conservation of the catalytic domain between both isoforms, it would not be surprising if many of the effects attributed to SGK1 in the brain turn out to be carried out by SGK1.1.

It has been proposed that SGK1 mediates the effects of glucocorticoids in the brain (22). Unlike SGK1, SGK1.1 does not seem to be a target of glucocorticoids but has high constitutive levels of expression in the CNS (7). However, the kinase still needs to be phosphorylated to become enzymatically active, a process that has been shown to be dependent on the PI3 kinase pathway for SGK1 (30). The region conserved between SGK1 and SGK1.1 includes the amino acid residues that are essential for kinase activation. Therefore, it is reasonable to assume that SGK1.1 activation will also depend on PI3 kinase activity. This idea is reinforced by the fact that SGK1.1 effects are enhanced by a phosphomimetic mutation in serine-515 (7), equivalent to serine-422 in SGK1, which is the primary target of the PI3 kinase activation pathway (30).

Taken together, the data available indicate that although SGK1 and SGK1.1 share many common properties, functional specificity is achieved by differential transcriptional regulation and subcellular localization. However, most of the studies addressing SGK1 transcriptional regulation and its effects on the activity of neuronal channels and transporters cannot differentiate between isoforms, and therefore a reevaluation of the relative importance of SGK1 and SGK1.1 in neuronal physiology is needed.

The physiological role of δ-ENaC in neurons is still uncertain. Voltage-independent, constitutively active Na^+ channels such as those formed by δ-ENaC could contribute to the resting Na^+ permeability of neurons. Recently, a member of the voltage-gated Na^+ channel family, NALCN, has been shown to form voltage-independent cation channels and encode the background Na^+ conductance in mouse hippocampal neurons (28). It is clear that regulation of such channels is essential for neuronal survival and excitability. If indeed δ-ENaC contributes to the resting Na^+ permeability of specific types of neurons such as the cortical pyramidal cells of the human and monkey cortex, the role of SGK1.1 could be essential in the maintenance and function of those neurons. Given the putative role of δ-ENaC in the transduction of ischemic signals during tissue inflammation and hypoxia (19), it is conceivable that SGK1.1 could also play a role in that signaling cascade.

The role of SGK1.1 as an integrator of signaling pathways. Addition of 3M3FBS, a specific activator of PLC (18), has been shown to produce translocation of SGK1.1 from the membrane to the cytoplasm, due to PtdIns(4,5)P_2 hydrolysis (7). Our experiments demonstrate that, in *Xenopus* oocytes, the kinase exhibits the same behavior when PLC is pharmacologically activated using 3M3FBS and additionally show the time course of SGK1.1 retrieval from the membrane. Moreover, we observed a 3M3FBS-induced decrease in δβγ-ENaC current only when the kinase is present, suggesting that PLC activation is able to trigger a cascade of signaling events that opposes the effects of SGK1.1.

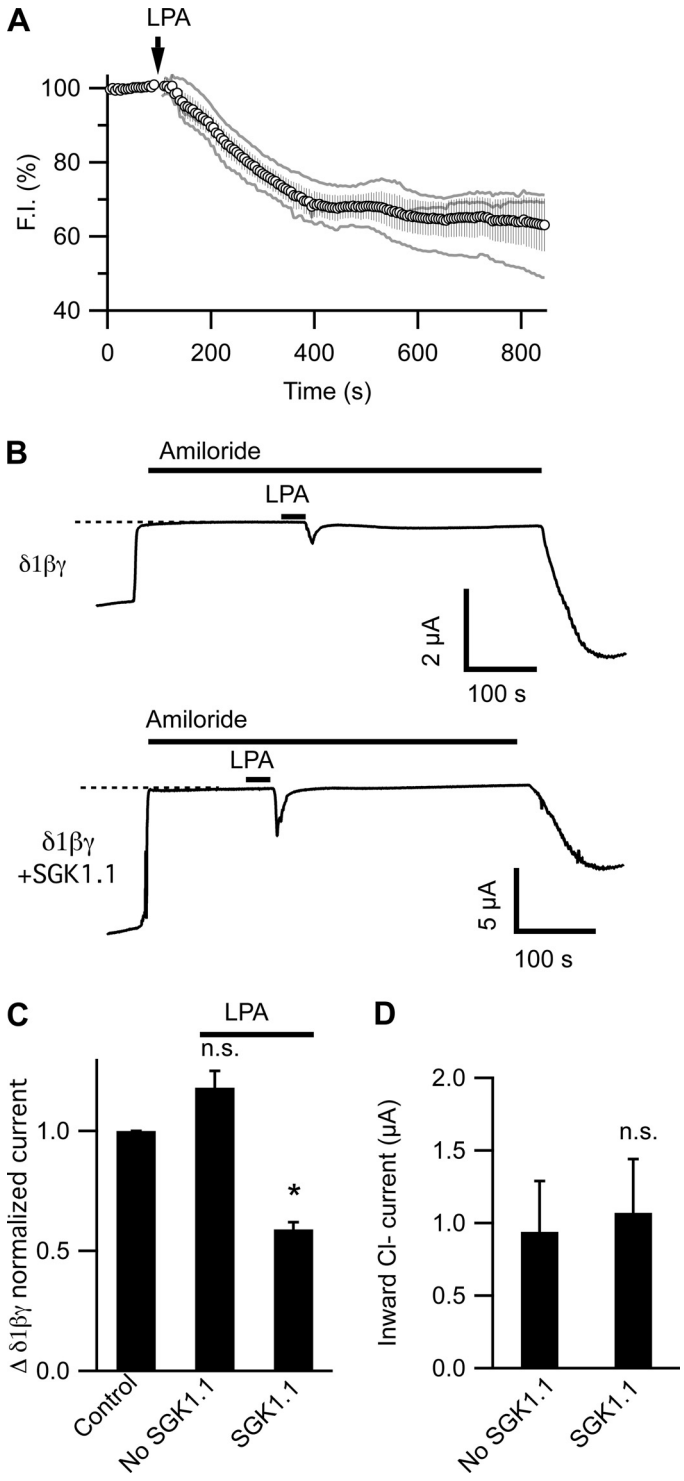

Fig. 7. Activation of PLC through lysophosphatidic acid (LPA) G protein-coupled receptors removes SGK1.1 from the membrane and diminishes its effect on δ-ENaC currents. *A*: average fluorescence intensity time course of oocytes expressing SGK1.1-CFP. Addition of 5 mM LPA is indicated with an arrow over the graph. Gray lines correspond to individual oocytes fluorescence time courses. Error bars represent SE ($n = 8$). *B*: representative current recordings of individual oocytes expressing δ1βγ alone (*top* trace) and δ1βγ with SGK1.1 (*bottom* trace). Addition of 100 μM amiloride and 5 mM LPA is shown with bars. Dotted line represents zero current. *C*: quantitative representation of amiloride-sensitive currents compared before and after addition of 5 mM LPA to oocytes expressing δ1βγ alone ($n = 7$) or δ1βγ with SGK1.1 ($n = 6$). *$P < 0.05$, Kruskal-Wallis nonparametric test followed by a Dunn's multicomparison test. *D*: average amount of Ca^{2+}-induced Cl^- currents activated after LPA addition, with or without SGK1.1. Not significant, Wilcoxon signed-rank test.

It is important to point out that direct regulation of αβγ-ENaC by PtdIns(4,5)P_2 hydrolysis has been previously described (23, 32). Interestingly, we do not observe any change of δβγ-ENaC current after 3M3FBS incubation, and only a significant current decrease is seen when SGK1.1 is coexpressed. The regulation of ENaC by PtdIns(4,5)P_2 hydrolysis could be specific of certain subunit combinations and/or cell types, and this is an interesting issue that should be pursued in more detail.

The validation of the *Xenopus* oocyte model allowed us to further demonstrate that the physiological PLC activation through a GPCR has a similar effect on SGK1.1 subcellular localization. Again, the shift in SGK1.1 localization abrogates its effects on δ channel activity. This effect takes place in a timeframe of minutes, consistent with a regulation of channel trafficking by the kinase, since it has been demonstrated that ENaC has a remarkably short half-life in the plasma membrane (4).

The probable need of PI3 kinase activity for SGK1.1 activation, together with its regulation by the PLC pathway, implies that this kinase has the potential to play a role as an integrator of different pathways converging on δ-ENaC activity in neurons. It has been shown that SGK1 serves an analogous role in kidney epithelial cells, integrating different hormonal signals (2, 31). In neurons, signals converging on SGK1.1 and δ-ENaC may include activators of PI3 kinase such as the brain-derived neurotrophic factor (1), which influences, among other processes, neuronal survival and plasticity (9). In addition, GPCR signaling through PLC, such as group I metabotropic glutamate receptors (8) or "M1-like" muscarinic receptors (16), could potentially modulate δ-ENaC activity in coordination with other pathways.

In summary, our results demonstrate that SGK1.1 provides a new mechanism of neuronal δ-ENaC regulation that is independent of the presence of accessory subunits and may act as a link between channel activity and phospholipase C signaling.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES

1. **Almeida RD, Manadas BJ, Melo CV, Gomes JR, Mendes CS, Graos MM, Carvalho RF, Carvalho AP, Duarte CB.** Neuroprotection by BDNF against glutamate-induced apoptotic cell death is mediated by ERK and PI3-kinase pathways. *Cell Death Differ* 12: 1329–1343, 2005.
2. **Alvarez de la Rosa D, Canessa CM.** Role of SGK in hormonal regulation of epithelial sodium channel in A6 cells. *Am J Physiol Cell Physiol* 284: C404–C414, 2003.
3. **Alvarez de la Rosa D, Canessa CM, Fyfe GK, Zhang P.** Structure and regulation of amiloride-sensitive sodium channels. *Annu Rev Physiol* 62: 573–594, 2000.
4. **Alvarez de la Rosa D, Paunescu TG, Els WJ, Helman SI, Canessa CM.** Mechanisms of regulation of epithelial sodium channel by SGK1 in A6 cells. *J Gen Physiol* 124: 395–407, 2004.
5. **Alvarez de la Rosa D, Zhang P, Naray-Fejes-Toth A. Fejes-Toth G, Canessa CM.** The serum and glucocorticoid kinase sgk increases the abundance of epithelial sodium channels in the plasma membrane of Xenopus oocytes. *J Biol Chem* 274: 37834–37839, 1999.
6. **Arteaga MF, Alvarez de la Rosa D, Alvarez JA, Canessa CM.** Multiple translational isoforms give functional specificity to serum- and glucocorticoid-induced kinase 1. *Mol Biol Cell* 18: 2072–2080, 2007.
7. **Arteaga MF, Coric T, Straub C, Canessa CM.** A brain-specific SGK1 splice isoform regulates expression of ASIC1 in neurons. *Proc Natl Acad Sci USA* 105: 4459–4464, 2008.
8. **Bird MK, Lawrence AJ.** Group I metabotropic glutamate receptors: involvement in drug-seeking and drug-induced plasticity. *Curr Mol Pharmacol* 2: 83–94, 2009.
9. **Bramham CR, Messaoudi E.** BDNF function in adult synaptic plasticity: the synaptic consolidation hypothesis. *Prog Neurobiol* 76: 99–125, 2005.
10. **Canessa CM, Schild L, Buell G, Thorens B, Gautschi I, Horisberger JD, Rossier BC.** Amiloride-sensitive epithelial Na^+ channel is made of three homologous subunits. *Nature* 367: 463–467, 1994.
11. **Debonneville C, Flores SY, Kamynina E, Plant PJ, Tauxe C, Thomas MA, Munster C, Chraibi A, Pratt JH, Horisberger JD, Pearce D, Loffing J, Staub O.** Phosphorylation of Nedd4–2 by Sgk1 regulates epithelial Na(+) channel cell surface expression. *EMBO J* 20: 7052–7059, 2001.
12. **Giraldez T, Afonso-Oramas D, Cruz-Muros I, Garcia-Marin V, Pagel P, Gonzalez-Hernandez T, Alvarez de la Rosa D.** Cloning and functional expression of a new epithelial sodium channel delta subunit isoform differentially expressed in neurons of the human and monkey telencephalon. *J Neurochem* 102: 1304–1315, 2007.
13. **Giraldez T, de la Peña P, Gomez-Varela D, Barros F.** Correlation between electrical activity and intracellular Ca^{2+} oscillations in GH3 rat anterior pituitary cells. *Cell Calcium* 31: 65–78, 2002.
14. **Giraldez T, Hughes TE, Sigworth FJ.** Generation of functional fluorescent BK channels by random insertion of GFP variants. *J Gen Physiol* 126: 429–438, 2005.
15. **Gonzalez MC, Abreu P, Barroso-Chinea P, Cruz-Muros I, Gonzalez-Hernandez T.** Effect of intracerebroventricular injection of lipopolysaccharide on the tuberoinfundibular dopaminergic system of the rat. *Neuroscience* 127: 251–259, 2004.
16. **Gulledge AT, Bucci DJ, Zhang SS, Matsui M, Yeh HH.** M1 receptors mediate cholinergic modulation of excitability in neocortical pyramidal neurons. *J Neurosci* 29: 9888–9902, 2009.
17. **Haerteis S, Krueger B, Korbmacher C, Rauh R.** The delta-subunit of the epithelial sodium channel (ENaC) enhances channel activity and alters proteolytic ENaC activation. *J Biol Chem* 284: 29024–29040, 2009.
18. **Horowitz LF, Hirdes W, Suh BC, Hilgemann DW, Mackie K, Hille B.** Phospholipase C in living cells: activation, inhibition, Ca^{2+} requirement, and regulation of M current. *J Gen Physiol* 126: 243–262, 2005.
19. **Ji HL, Benos DJ.** Degenerin sites mediate proton activation of delta-beta-gamma-ENaC. *J Biol Chem* 279: 26939–26947, 2004.
20. **Ji HL, Su XF, Kedar S, Li J, Barbry P, Smith PR, Matalon S, Benos DJ.** Delta-subunit confers novel biophysical features to alpha beta gamma-human epithelial sodium channel (ENaC) via a physical interaction. *J Biol Chem* 281: 8233–8241, 2006.
21. **Kamynina E, Debonneville C, Bens M, Vandewalle A, Staub O.** A novel mouse Nedd4 protein suppresses the activity of the epithelial Na^+ channel. *FASEB J* 15: 204–214, 2001.
22. **Kaufer D, Ogle WO, Pincus ZS, Clark KL, Nicholas AC, Dinkel KM, Dumas TC, Ferguson D, Lee AL, Winters MA, Sapolsky RM.** Restructuring the neuronal stress response with anti-glucocorticoid gene delivery. *Nat Neurosci* 7: 947–953, 2004.
23. **Kunzelmann K, Bachhuber T, Regeer R, Markovich D, Sun J, Schreiber R.** Purinergic inhibition of the epithelial Na^+ transport via hydrolysis of PIP_2. *FASEB J* 19: 142–143, 2005.

24. **Lang F, Artunc F, Vallon V.** The physiological impact of the serum and glucocorticoid-inducible kinase SGK1. *Curr Opin Nephrol Hypertens* 18: 439–448, 2009.
25. **Lang F, Bohmer C, Palmada M, Seebohm G, Strutz-Seebohm N, Vallon V.** (Patho)physiological significance of the serum- and glucocorticoid-inducible kinase isoforms. *Physiol Rev* 86: 1151–1178, 2006.
26. **Lassmann T, Sonnhammer EL.** Kalign, Kalignvu and Mumsa: web servers for multiple sequence alignment. *Nucleic Acids Res* 34: W596–W599, 2006.
27. **Liu W, Yuen EY, Yan Z.** The stress hormone corticosterone increases synaptic alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors via serum- and glucocorticoid-inducible kinase (SGK) regulation of the GDI-Rab4 complex. *J Biol Chem* 285: 6101–6108, 2010.
28. **Lu B, Su Y, Das S, Liu J, Xia J, Ren D.** The neuronal channel NALCN contributes resting sodium permeability and is required for normal respiratory rhythm. *Cell* 129: 371–383, 2007.
29. **McNicholas CM, Canessa CM.** Diversity of channels generated by different combinations of epithelial sodium channel subunits. *J Gen Physiol* 109: 681–692, 1997.
30. **Park J, Leong ML, Buse P, Maiyar AC, Firestone GL, Hemmings BA.** Serum and glucocorticoid-inducible kinase (SGK) is a target of the PI 3-kinase-stimulated signaling pathway. *EMBO J* 18: 3024–3033, 1999.
31. **Pearce D.** SGK1 regulation of epithelial sodium transport. *Cell Physiol Biochem* 13: 13–20, 2003.
32. **Pochynyuk O, Bugaj V, Stockand JD.** Physiologic regulation of the epithelial sodium channel by phosphatidylinositides. *Curr Opin Nephrol Hypertens* 17: 533–540, 2008.
33. **Raikwar NS, Snyder PM, Thomas CP.** An evolutionarily conserved N-terminal Sgk1 variant with enhanced stability and improved function. *Am J Physiol Renal Physiol* 295: F1440–F1448, 2008.
34. **Ruffieux-Daidie D, Poirot O, Boulkroun S, Verrey F, Kellenberger S, Staub O.** Deubiquitylation regulates activation and proteolytic cleavage of ENaC. *J Am Soc Nephrol* 19: 2170–2180, 2008.
35. **Snyder PM, Olson DR, Thomas BC.** Serum and glucocorticoid-regulated kinase modulates Nedd4–2-mediated inhibition of the epithelial Na^+ channel. *J Biol Chem* 277: 5–8, 2002.
36. **Staub O, Dho S, Henry P, Correa J, Ishikawa T, McGlade J, Rotin D.** WW domains of Nedd4 bind to the proline-rich PY motifs in the epithelial Na^+ channel deleted in Liddle's syndrome. *EMBO J* 15: 2371–2380, 1996.
37. **Strutz-Seebohm N, Seebohm G, Shumilina E, Mack AF, Wagner HJ, Lampert A, Grahammer F, Henke G, Just L, Skutella T, Hollmann M, Lang F.** Glucocorticoid adrenal steroids and glucocorticoid-inducible kinase isoforms in the regulation of GluR6 expression. *J Physiol* 565: 391–401, 2005.
38. **Van-Ham I, Lupu-Meiri M, Tayer M, Shapira H, Oron Y.** Response to lysophosphatidic acid in *Xenopus* oocytes and its rapid desensitization: the role of Gq and Go G-protein families. *J Cell Physiol* 200: 125–133, 2004.
39. **Vuagniaux G, Vallet V, Jaeger NF, Hummler E, Rossier BC.** Synergistic activation of ENaC by three membrane-bound channel-activating serine proteases (mCAP1, mCAP2, and mCAP3) and serum- and glucocorticoid-regulated kinase (Sgk1) in *Xenopus* oocytes. *J Gen Physiol* 120: 191–201, 2002.
40. **Waldegger S, Barth P, Raber G, Lang F.** Cloning and characterization of a putative human serine/threonine protein kinase transcriptionally modified during anisotonic and isotonic alterations of cell volume. *Proc Natl Acad Sci USA* 94: 4440–4445, 1997.
41. **Waldmann R, Champigny G, Bassilana F, Voilley N, Lazdunski M.** Molecular cloning and functional expression of a novel amiloride-sensitive Na+ channel. *J Biol Chem* 270: 27411–27414, 1995.
42. **Webster MK, Goya L, Ge Y, Maiyar AC, Firestone GL.** Characterization of sgk, a novel member of the serine/threonine protein kinase gene family which is transcriptionally induced by glucocorticoids and serum. *Mol Cell Biol* 13: 2031–2040, 1993.
43. **Yamamura H, Ugawa S, Ueda T, Nagao M, Shimada S.** A novel spliced variant of the epithelial Na^+ channel delta-subunit in the human brain. *Biochem Biophys Res Commun* 349: 317–321, 2006.