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The Serum and Glucocorticoid Kinase *sgk* Increases the Abundance of Epithelial Sodium Channels in the Plasma Membrane of *Xenopus* Oocytes*

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The serum- and glucocorticoid-induced kinase (*sgk*) is a serine and threonine kinase that stimulates amiloride-sensitive sodium transport in *Xenopus* oocytes. Because aldosterone induces phosphorylation on serine/threonine (Ser/Thr) residues in the carboxyl termini of β and γ subunits of epithelial sodium channels (ENaCs) and causes an increase in the *sgk* transcript in mammalian and amphibian renal epithelial cells, it seems likely that *sgk* mediates the action of aldosterone to stimulate sodium transport. Experiments were performed in *Xenopus* oocytes to determine the mechanism by which *sgk* increases sodium conductance by examining its effect on phosphorylation, kinetics, and membrane abundance of ENaC. Our results demonstrate that deletions of the carboxyl termini of the three subunits do not inhibit *sgk*-induced sodium current, indicating that the effect of *sgk* is not mediated via phosphorylation within the carboxyl termini of ENaC. They also show no evidence that *sgk* reduces the removal of ENaC from the plasma membrane because mutations of tyrosine residues in the sequences necessary for endocytosis and degradation did not affect the response to *sgk*. Further studies performed with the patch-clamp technique indicated that *sgk* did not increase the open probability or changed the kinetics of ENaC. These studies, however, showed a 3-fold increase in the abundance of ENaC in the plasma membrane in the presence of *sgk* compared with control. Together, the experiments indicate that *sgk* stimulates electrogenic sodium transport by increasing the number of ENaCs at the cell surface and suggest that *sgk* may mediate the early increase in aldosterone-induced sodium current.

The cortical collecting tubule of the kidney exhibits a rapid increase in sodium permeability after stimulation with aldosterone (1). The initial increase in sodium permeability occurs prior to the synthesis of new channels (2, 3). In contrast, chronic aldosterone treatment results in the synthesis of many proteins involved in sodium reabsorption, including the subunits of ENaC,[1] Na^+/K^+-ATPase, and mitochondrial enzymes (1, 4, 5). Therefore, the increase in sodium permeability seen in the early phase might be mediated by activation of silent channels or by insertion of preformed channels. Recent findings suggest that *sgk* may be involved in the early phase of the action of aldosterone. *sgk* (serum- and glucocorticoid-induced kinase) is a novel member of the serine and threonine kinase gene family (6). The activity of *sgk* is regulated by rapid induction of transcription of its mRNA. Depending on the cell type, *sgk* expression is induced by glucocorticoids (6), follicle-stimulating hormone (7), cell volume changes (8, 9), and aldosterone (10, 11). In the cortical collecting tubule of the rat and rabbit (10) and in the A6 cell line derived from the distal tubule of the kidney of *Xenopus laevis* (11), aldosterone increases the levels of *sgk* mRNA and protein within 30 min after addition of the hormone. This rapid induction suggests that *sgk* may mediate the early increase of sodium permeability characteristically observed in the cortical collecting tubule after stimulation with aldosterone.

Because *sgk* is a Ser/Thr kinase, the first possibility to consider is whether *sgk* phosphorylates ENaC. This possibility was raised by previous studies demonstrating that the carboxyl termini of β and γ subunits of ENaC are phosphorylated when the channels are expressed in Madin-Darby canine kidney cells (a cell line derived from canine distal tubule of the kidney) (12). Phosphorylation of ENaC could increase the open probability or the insertion of preformed channels and/or decrease the retrieval. Alternatively, the effect of *sgk* could involve phosphorylation of other proteins that modify the activity of ENaC or its abundance in the plasma membrane.

In this study, we examined the basic mechanism by which *sgk* increases sodium permeability. We used *Xenopus* oocytes co-injected with cRNAs encoding ENaC and *sgk* as our experimental model. This strategy provides an approach to examine the activity of channels with electrophysiological techniques and also to measure expression of channel proteins with biochemical methods. The validity of this model system is based on the observation that *sgk* increases the magnitude of amiloride-sensitive currents in oocytes (10, 11), indicating that these cells have the machinery necessary to mediate the *sgk* response.

MATERIALS AND METHODS

cDNA Constructs—cDNAs from rat ENaC subunits were used in all experiments. Deletions of the carboxyl termini to generate truncated subunits α_T, β_T, and γ_T and replacement of tyrosine for alanine residues to generate αY673A, βY618A, and γY628A have been previously de-

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[1] The abbreviations used are: ENaC, epithelial sodium channel; BFA, brefeldin A.

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scribed (13). Substitutions of six serine and threonine residues (T570A/T575A/T584A/T613A/S620A/S631A) in the carboxyl terminus of the β subunit for alanines ($\beta 6PO_4$), and of five in the amino terminus of the α subunit (S80A/T92A/T93A/S103A/T110A) were made by polymerase chain reaction. Presence of the mutations in the cDNAs was confirmed by DNA sequencing. The FLAG epitope was introduced in the α subunit by substituting residues Thr^{194} to Arg^{201} with the sequence DYKDDDDK (α^{FLAG}) (14). The mouse *sgk*/pSP64poly(A) construct was generated as described earlier (10). cRNAs were made from linearized plasmids using the mMessagemMachine kit (Ambion, Austin, TX) according to the manufacturer's instructions.

Oocyte Isolation and cRNA Injection—Xenopus laevis oocytes were surgically removed from adult female frogs using standard procedures. Stage V–VI oocytes were injected with 1 ng of cRNA from each of the wild-type or mutant subunits in the following combinations: α, β, and γ; α and β; α and γ; or α alone. When indicated, 1 ng of *sgk* cRNA was also injected. Oocytes were incubated at 19 °C for 1–3 days in amphibian Ringer supplemented with 10 μM amiloride.

Electrophysiology and Data Evaluation—Electrophysiological recordings were performed using two-electrode voltage clamp and patch clamp techniques. Current and voltage electrodes were pulled from borosilicate glass and filled with 3 M KCl. Electrode resistances were <1 MΩ. Epithelial sodium channel currents were calculated as the difference in whole-cell current before and after the addition of 50 μM amiloride to the bathing solution. Currents were recorded with an OC-725B oocyte voltage clamp (Warner Instrument Corp., Hamden, CT) and digitized at 0.1 kHz (ITC-16), and the values were stored on the hard drive of a personal computer. Membrane potential was held at -60 mV. Current-voltage relations were generated by changing the membrane potential from -180 to 80 mV in 20 mV incremental steps of 200 ms duration using Pulse (HEKA, Lamprecht, Germany). I–V curves were fitted to the constant field equation. The composition of the standard bath solution was 100 mM sodium gluconate, 4 mM KCl, 2 mM $CaCl_2$, 10 mM HEPES, pH adjusted to 7.4 with KOH. Single channel recordings were made from cell-attached and inside-out patches. For patch-clamp recordings, pipette-to-membrane seals with resistances of 9–15 GΩ were formed with pipettes made from borosilicate capillary glass by a two-stage pulling and fire-polishing process. The composition of the pipette solution was 150 mM LiCl, 1 mM $CaCl_2$, 10 mM Tris buffered to pH 7.4, and the composition of the bath was 150 mM KCl, 5 mM EDTA, 10 mM HEPES, pH adjusted to 7.4 with KOH. An Axopatch 200B amplifier and Digidata 1200B (Axon Instruments, Forster City, CA) interfaced to a personal computer were used to acquire data at 5 kHz. The data were filtered at 100 Hz during acquisition using an eight-pole Bessel filter (Frequency Devices, Inc., Haveford, MA) and stored directly on the hard drive of a personal computer. Open probability was calculated from single- and multichannel patches using pClamp7. Lists of open- and closed-current intervals were generated via a half-amplitude threshold crossing criterion using Fetchan. Only patches containing one channel were used to determine open and closed times, and all events, independent of duration, were included in the analysis. For this purpose, single-channel data were digitally filtered (Gaussian) at 200 Hz. Histograms were generated with data from several patches. The fitting method was simplex-least squares. Results are expressed as mean $\pm$ S.E. Differences between groups were assessed using Student's *t* test, and $p < 0.05$ was considered to be statistically significant.

Metabolic Labeling of Oocytes—Oocytes injected with ENaC alone or with ENaC and *sgk* were labeled for 8 h with 0.5 mCi/ml of a mixture of $[^{35}S]$methionine and cysteine (*in vitro* cell labeling mix, Amersham Pharmacia Biotech). After labeling, cells were homogenized in 1% Triton X-100 and evaluated for total protein and specific activity. An equal number of counts were immunoprecipitated with anti-α, -β, and -γ specific antibodies. A 50-μl aliquot of protein A slurry was added to each sample to isolate the immune complexes. After washes with homogenization buffer, the products were resolved by 10% SDS-polyacrylamide gel electrophoresis. Gels were treated with 1 M sodium salicylate, dried, and exposed to x-ray film for fluorography.

Labeling of Surface Channels from Oocytes—Oocytes were injected with α^{FLAG} cRNA and wild-type β and γ cRNAs, with or without *sgk* cRNA. After 36 h, cells were incubated on ice with 10% bovine serum albumin for 30 min. 25 nM/100 μl anti-FLAG M2 monoclonal antibody (Kodak) was added, and the incubation continued for 1 h. After 10 washes with ice-cold amphibian Ringer solution, cells were incubated with ^{125}I-protein G (1 μCi/100 μl, 1 nM/100 μl) (ICN Biomedicals, Inc. Irvine, CA). Oocytes were washed with 1 ml of ice-cold amphibian Ringer 10 times. Three oocytes were placed in each tube for measurements of gamma counts. Water-injected oocytes served as controls in these experiments. Results are expressed as mean $\pm$ S.E.

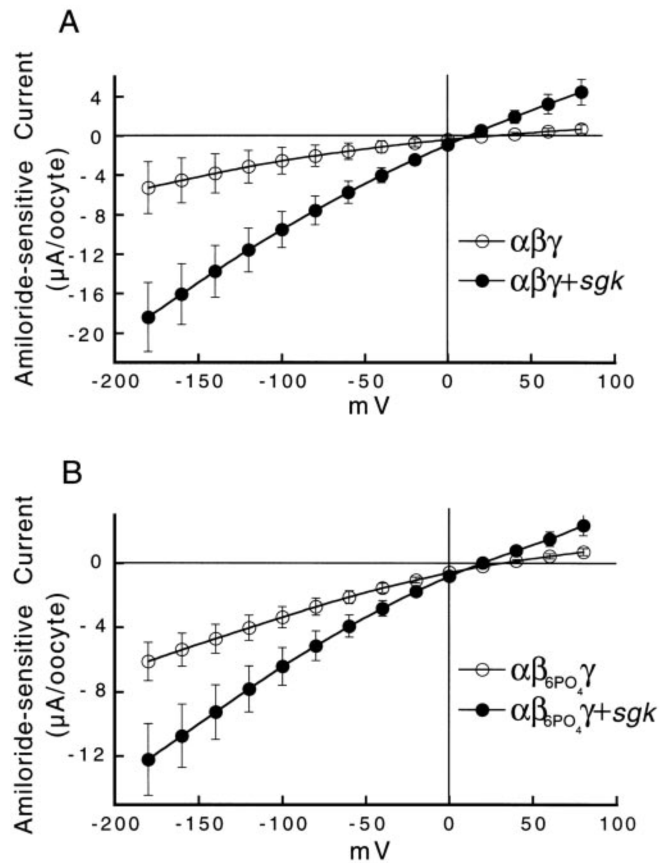

FIG. 1. **I–V curves from oocytes injected with wild-type $\alpha\beta\gamma$ channels and $\alpha\beta 6PO_4\gamma$ channels mutated in six conserved Ser/Thr residues in the carboxyl terminus of the β subunit.** *A,* wild-type $\alpha\beta\gamma$ without and with *sgk*. *B,* channels with mutant $\beta 6PO_4$ subunit without and with *sgk*. *Data points* represent the amiloride-sensitive component of whole-cell currents in the presence of 100 mM Na^+ in the bath. *Lines* are the fits of the data to the constant field equation. Each *data point* is the mean of 30–40 oocytes. *Error bars* are S.E. The values from the two conditions were statistically different, with $p < 0.01$.

RESULTS

Role of Serine and Threonine Residues in the Carboxyl Terminus of β Subunits in the Response to sgk—We have previously shown that ENaCs are phosphorylated in the carboxyl termini of the β and γ subunits under basal conditions and that aldosterone induces additional phosphorylation mainly in the β subunit in transfected Madin-Darby canine kidney cells (12). Because *sgk* activates *Xenopus* and rat ENaCs, the most likely candidates for *sgk* phosphorylation are serine and threonine residues conserved in the β subunits from both species. We identified six conserved residues in the sequence of the rat β subunit: Thr-570, Thr-575, Thr-584, Thr-613, Ser-620, and Ser-631. These six Ser/Thr residues were replaced by alanines to generate the mutant $\beta 6PO_4$. Oocytes were injected with wild-type α and γ subunits and mutant $\beta 6PO_4$ with and without *sgk* cRNA. 36 h after injection, the activity of channels was examined by measuring amiloride-sensitive whole-cell currents with the two-electrode voltage clamp. Fig. 1 shows the I–V curves from oocytes expressing wild-type $\alpha\beta\gamma$ channels with or without *sgk* and mutated $\alpha\beta 6PO_4\gamma$ channels with or without *sgk*. The mean currents in oocytes expressing wild-type or mutant channels were of the same magnitude: 2.53 $\pm$ 1.35 μA/oocyte in the wild-type and 3.35 $\pm$ 0.65 μA/oocyte in the mutant group (values taken at a membrane potential of -100 mV). Coexpression with *sgk* increased the current of wild-type channels to 9.45 $\pm$ 1.35 and of mutant channels to 6.42 $\pm$ 1.17 μA/oocyte, which represents a 3.7- and 2.9-fold increase in current, respec-

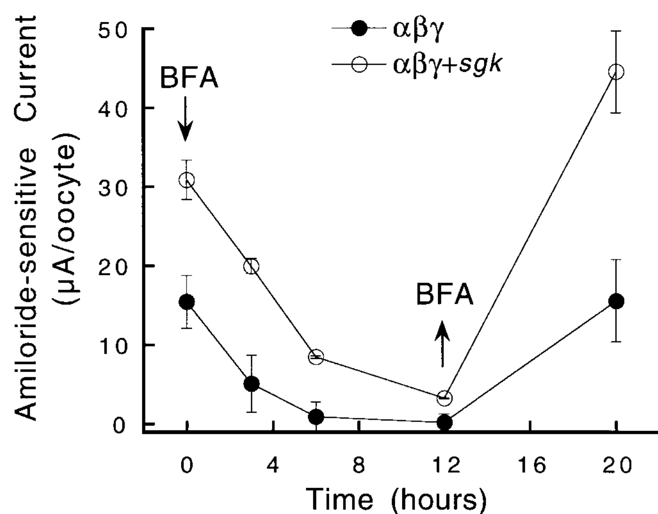

FIG. 2. **Rate of retrieval of channels from the plasma membrane.** Oocytes were injected with $\alpha\beta\gamma$ alone or with *sgk* cRNAs. 36 h later, 5 μM BFA was added to the incubation medium, and whole-cell currents were measured at 3-h intervals with the two-electrode voltage clamp. *Arrows* indicate the times of addition and removal of BFA. Each *symbol* represents the mean of 8–10 oocytes; *error bars* are S.E.

tively. Although the response of mutant channels was slightly smaller, the difference was not statistically significant. These results indicate that *sgk*-induced sodium permeability is not mediated by phosphorylation of conserved serine and threonine residues in the carboxyl terminus of the β subunit. In addition, these six Ser/Thr residues are not required to maintain basal channel activity because wild-type and mutant channels exhibited the same level of current.

sgk and Retrieval of Channels from the Plasma Membrane—In addition to the six Ser/Thr residues mutated in the β subunit, the carboxyl termini of the three subunits contain other potential phosphorylation sites, as well as PY sequences involved in interactions with the ubiquitin ligase Nedd4 (15, 16) and with components from the endocytic machinery (13). Ubiquitination of the subunits by Nedd4 and clathrin-mediated endocytosis both promote retrieval of channels from the plasma membrane. *sgk* could disrupt interaction of the channel with these proteins by phosphorylation of residues in this domain or by associating with the carboxyl termini of the subunits. To test whether *sgk* slows the rate of endocytosis of channels from the plasma membrane, we treated oocytes with 5 μM brefeldin A (BFA) and followed the levels of amiloride-sensitive currents over a period of several hours with the two-electrode voltage clamp (13). BFA inhibits the secretory pathway by blocking anterograde vesicular transport from the endoplasmic reticulum and delivery of newly synthetized channels to the plasma membrane. In the presence of BFA, the decrease of amiloride-sensitive currents reflects the rate of removal of channels from the plasma membrane. We added BFA to the incubation medium of oocytes expressing ENaC ± *sgk* and measured whole-cell currents at 3-h intervals in both groups. Currents decreased at a similar rate in oocytes expressing *sgk* (Fig. 2), suggesting that endocytosis of ENaC is not affected by *sgk*. Furthermore, because the PY motifs have been shown to be necessary for removal of channels from the cell surface (13, 14), we also examined whether mutations or deletions of these motifs from α, β, and γ subunits could affect the response to *sgk*. Channels with mutations in all three tyrosine residues ($\alpha_{Y673A}\beta_{Y618A}\gamma_{Y628A}$) and with deletions of the carboxyl termini ($\alpha_T\beta_T\gamma_T$) were expressed with and without *sgk*. As expected, oocytes injected with $\alpha_{Y673A}\beta_{Y618A}\gamma_{Y628A}$ (5.66 ± 0.4 μA/oocyte) and $\alpha_T\beta_T\gamma_T$ (14 ± 1.26 μA/oocyte) expressed larger currents

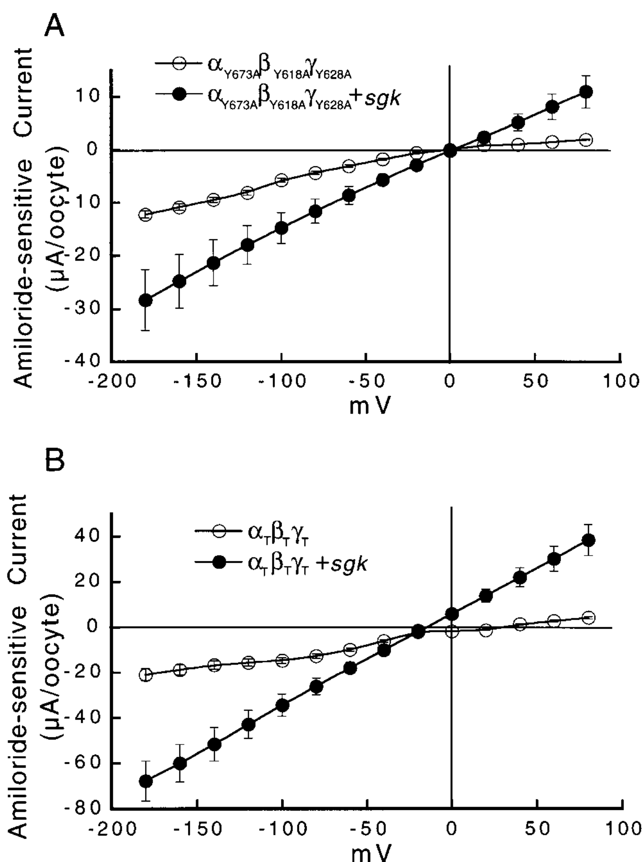

FIG. 3. **Stimulation of amiloride-sensitive currents by *sgk* in oocytes injected with channels mutated in the PY motifs or with deletions of the carboxyl termini from all three subunits.** *A,* I–V curves of oocytes injected with $\alpha_{Y673A}\beta_{Y618A}\gamma_{Y628A}$ channels alone or with *sgk*. *B,* I–V curves of oocytes injected with $\alpha_T\beta_T\gamma_T$ channels alone or with *sgk*. Each *data point* represents the mean of 20–30 oocytes. *Error bars* are S.E. $p < 0.01$.

than oocytes injected with wild-type channels. Most significantly, $\alpha_{Y673A}\beta_{Y618A}\gamma_{Y628A}$ and $\alpha_T\beta_T\gamma_T$ channels responded to sgk with a further increment in current: 14.6 ± 2.9 and 34.2 ± 4.9 μA/oocyte, respectively (Fig. 3).

Effect of sgk on ENaCs with Different Subunit Compositions—Amiloride-sensitive Na^+ channels with distinct functional properties are generated by various combinations of the α, β and γ subunits: $\alpha\beta\gamma$, $\alpha\beta$, $\alpha\gamma$, and α alone (17). To determine which subunits are required for the response to *sgk*, we expressed α, $\alpha\beta$, and $\alpha\gamma$ channels with and without *sgk*. As previously reported, α, $\alpha\beta$, and $\alpha\gamma$ channels expressed current smaller than that of $\alpha\beta\gamma$ (18) but large enough for accurate measurements. All these three types of channels responded to *sgk* with an increase in current. In oocytes expressing α channels, the current increased from 20 ± 3 to 180 ± 25 nA/oocyte, in $\alpha\gamma$ channels from 0.5 ± 0.12 to 1.37 ± 0.19 μA/oocyte, and in $\alpha\beta$ channels from 2 ± 0.22 to 10 ± 2.2 μA/oocyte. These mean values were taken at −60 mV; currents at different voltages are shown in the I–V curves in Fig. 4.

These results provide important information regarding the mechanism of channel activation by *sgk*. According to one of our previous reports (18), the mean open probability (P_o) of $\alpha\gamma$ channels is ~0.5, whereas the P_o of $\alpha\beta$ channels is close to 1. Because the P_o of $\alpha\beta$ channels is near unity, the increase in current induced by *sgk* could be accounted for only by an increase in the number of active channels in the plasma membrane and not by changes in P_o.

In addition, the data indicate that β and γ subunits are not required for the response to *sgk* because oocytes expressing

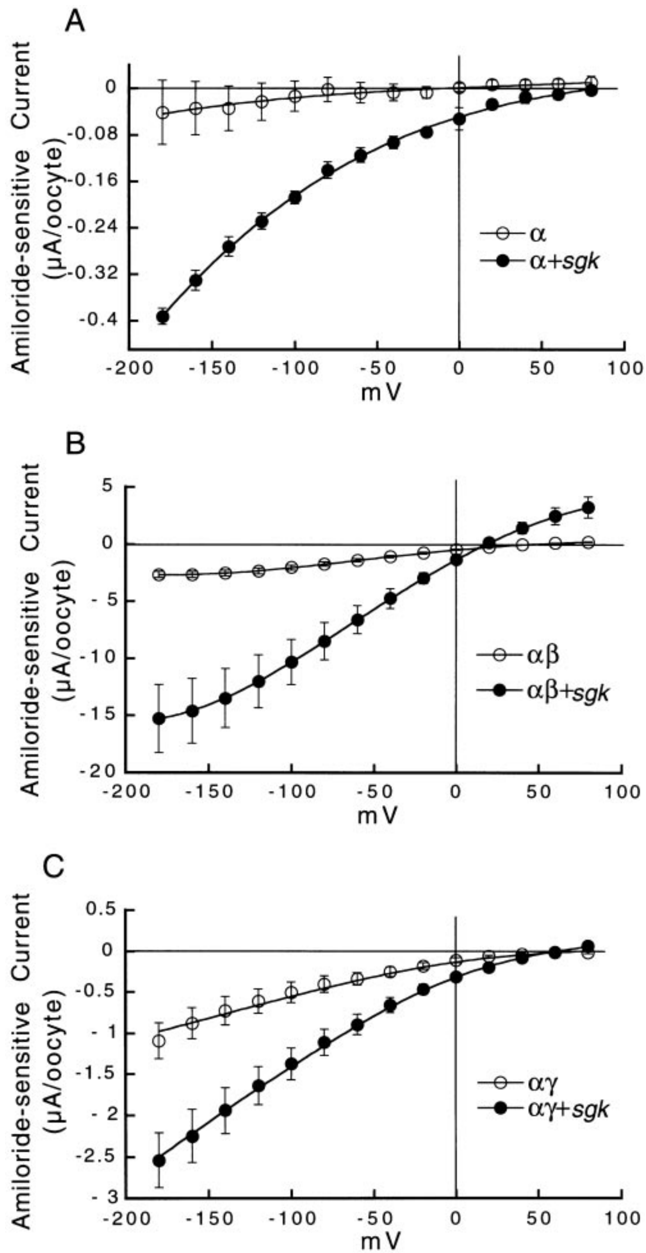

FIG. 4. **Effect of *sgk* on Na^+ currents from oocytes expressing α, αβ and αγ ENaCs.** I–V curves represent the amiloride-sensitive component of whole-cell currents from oocytes expressing α, αβ, or αγ channels with and without *sgk*. $n = 20–30$ oocytes. *Error bars* are S.E. $p < 0.001$.

only α subunits exhibited a similar increase in currents. Within the α subunits, the carboxyl terminus is also not necessary, as we showed in the experiments in Fig. 3*B*. To investigate whether the amino terminus from α subunits could be phosphorylated by *sgk,* we made a construct in which the first 77 residues were deleted. However, deletion of most of the amino terminus from α completely inactivated the channels. Mutations of five Ser and Thr residues (S80A/T92A/T93A/S103A/T110A) common to the rat and *Xenopus* α sequences also produced inactive channels.

Measurements of P_o and Characterization of the Kinetics of αβγ ENaCs Coexpressed with sgk—The previous experiments strongly suggest that *sgk* does not increase the P_o of αβ channels. To further investigate the effect of *sgk* on the kinetics of ENaC, we examined the P_o and kinetics of αβγ channels alone and in the presence of *sgk* with the patch clamp technique. The

TABLE I
Values of the mean open probability (P_o) and mean open (τ_o) and closed times (τ_c) from αβγ expressed alone and with sgk

P_o was calculated from patches containing 1–5 channels using the procedure described under "Materials and Methods." The mean open and closed times were calculated from dwell-time histograms of open and closed events constructed with data from patches containing single channels. Time constants were obtained by fitting the data to single exponential functions for the τ_o and the sum of two exponentials for τ_c. All values are the mean ± S.E.

	αβγ	αβγ + *sgk*
Open probability	0.83 ± 0.031	0.87 ± 0.04
τ_o (ms)	1309	1865
τ_c short (ms)	5.8	5.2
τ_c long (ms)	266	356

mean P_o was calculated from at least 16–18 patches for each condition. Single- and multichannel patches were included in this analysis. Data were collected under identical experimental conditions for both groups: the same batch of oocytes was injected with αβγ or with αβγ and *sgk*, and recordings were made the same day from both groups.

The mean P_o of αβγ channels was 0.83 ± 0.031, and in the presence of *sgk*, the P_o was 0.87 ± 0.04 (Table I). The mean open (τ_o) and closed (τ_c) times were determined with data collected from patches containing only single channels. Histograms were constructed with data from several patches in order to accumulate enough number of events. Fig. 5 shows the frequency distributions of the open and closed dwell times of αβγ channels expressed alone and with *sgk*. Histograms of the open dwell times were well fitted with a single exponential probability density function. The time constants of the open states were 1309 ms for αβγ channels and 1865 ms for channels coexpressed with *sgk*. In contrast, the closed dwell time histograms were best fitted with two exponentials. The τ_c of the short closed state of αβγ channels was 5.8 ms, and the τ_c of the long close estate was 266 ms. The two τ_c of αβγ channels with *sgk* were 5.2 and 356 ms, respectively (Fig. 5 and Table I). The closed times with and without *sgk* were not statistically different for the two groups.

On several occasions, channels were initially recorded in the cell-attached configuration for 5–10 min, and then they were excised and the recording continued for 5–10 min. Finally, the patches were reintroduced into the cytoplasm of the oocyte and recorded for an additional 5–10 min. In these experiments, we did not detect significant changes in the P_o or in the kinetics of channels when the patches were excised or after reestablishing contact with the cytoplasm. These observations strengthen the notion that *sgk* does not modify the P_o of ENaC.

Abundance of the Subunits of ENaC in Oocytes Expressing sgk—Results of the previous experiments are consistent with *sgk* increasing the number of channels at the cell surface without changing the P_o. Such an effect could be produced by enhancing the synthesis and insertion of subunits, thereby increasing the number of channels in the cell. To investigate this possibility, we estimated the amount of ENaC subunits by immunoprecipitation with specific anti-ENaC antibodies in oocytes expressing channels only or with *sgk*. Oocytes were labeled with [^{35}S]methionine and cysteine for 8 h. After determining the specific activity of the labeled proteins, equal amounts of counts were immunoprecipitated from each condition, and the products resolved by SDS-polyacrylamide gel electrophoresis. The results of these experiments are illustrated in the gels shown in Fig. 6. Quantification of the intensity of the bands revealed similar levels of expression of each of the subunits in the two groups.

Expression of Channels at the Cell Surface—Although *sgk* does not change the amount of subunits in oocytes, we cannot

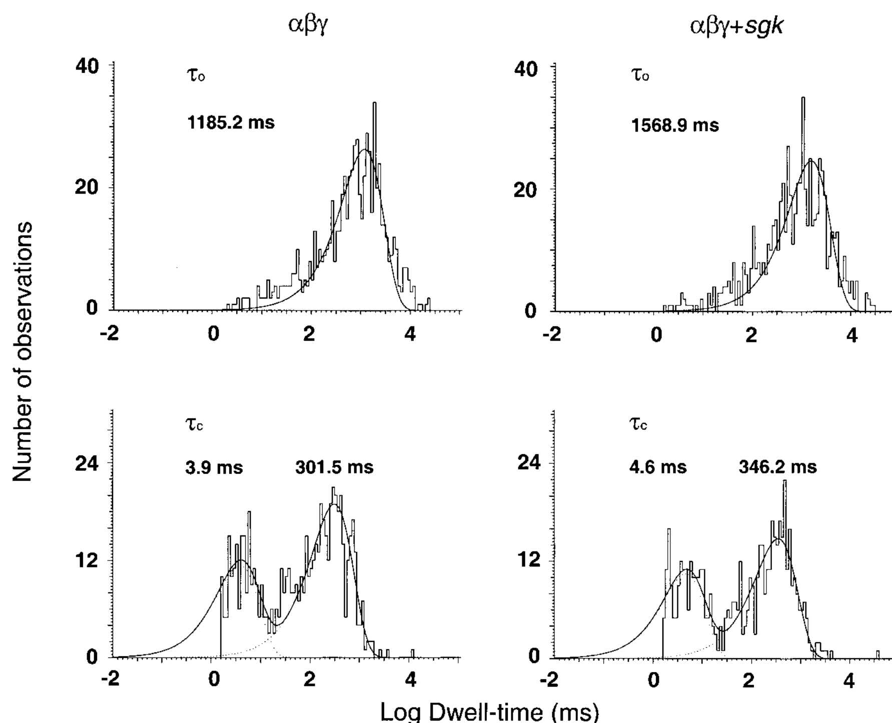

FIG. 5. **Dwell time histograms of open and closed events recorded from $\alpha\beta\gamma$ channels expressed alone or with *sgk*.** For the open events (*upper panel*), the *solid lines* in the histograms represent the fit of each data set with a single exponential probability density function. The *solid lines* of the closed event histograms (*lower panel*) represent the fits to a sum of two exponential functions with a short and a long time constant. The time constants (ms) are shown for the open (τ_0) and closed (τ_c) states. For each condition, the data were collected from nine single channel patches, each patch recorded for at least 5 min.

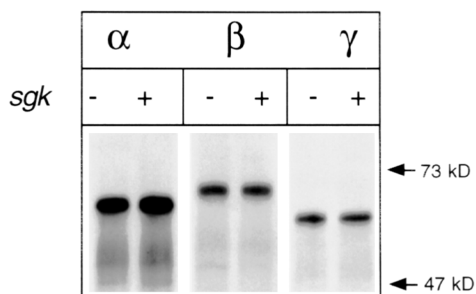

FIG. 6. **Immunoprecipitations of α, β, and γ subunits from oocytes expressing only ENaC or with *sgk*.** Oocytes were injected with 1 ng of cRNA from each of the subunits and were labeled with [^{35}S]methionine for 8 h. Equal amounts of radioactive counts were immunoprecipitated from each group with specific antibodies for α, β, and γ subunits. Immune complexes were resolved by 10% SDS-polyacrylamide gel electrophoresis.

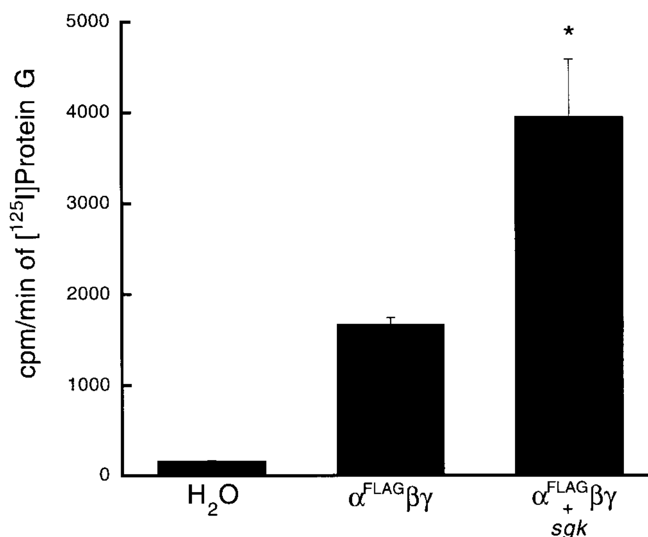

FIG. 7. **Expression of channels at the cell surface.** Oocytes were injected with water, $\alpha^{FLAG}\beta\gamma$, or $\alpha^{FLAG}\beta\gamma$ with *sgk*. Cells were incubated with anti-FLAG monoclonal antibody for 1 h on ice and washed, and ^{125}I-protein G was added for 1 h. Each *data point* represents counts measured from three oocytes. *Columns* and *error bars* are the mean ± S.E. of 24 independent measurements from each group. *, statistically significant difference ($p < 0.001$).

exclude a selective increase in the number of channels at the cell surface, because only a small fraction of the total number of channels is expressed in the plasma membrane of oocytes (19). In order to assess the number of channels in the plasma membrane, we introduced a FLAG epitope in the extracellular domain of the α subunit in the same manner as reported previously by Firsov *et al.* (14). Oocytes were injected with $\alpha^{FLAG}\beta\gamma$ alone or in combination with *sgk,* and after 36 h, intact cells were incubated with an anti-FLAG monoclonal antibody. Antibodies reacting with α^{FLAG} on the surface of oocytes were detected with ^{125}I-protein G. Groups of three oocytes were measured as a single data point. The radioactivity from the three experimental conditions, $\alpha\beta\gamma$ alone, $\alpha\beta\gamma$ with *sgk*, and water-injected oocytes, is shown in Fig. 7. Oocytes expressing *sgk* had 3-fold more counts than oocytes injected with channels alone, reflecting a larger number of α subunits expressed at the cell surface.

DISCUSSION

The results of this work demonstrate that *sgk*, an aldosterone-induced Ser/Thr kinase in collecting duct cells (10), activates ENaC by increasing the number of channels in the plasma membrane. The effect is not achieved by reducing the rate of endocytosis, as is the case for mutations causing Liddle's syndrome, but rather by insertion of channels into the plasma membrane. *sgk* did not change the P_o or kinetics of channels. This conclusion was reached from several different experiments. With the patch clamp technique, we determined that the P_o of wild-type channels was high in oocytes, and the kinetics were characterized by long openings of several seconds' duration and two closed states, a short one of less than 10 ms and a longer one in the range of 200–300 ms. Expression with *sgk* did not change the P_o or the kinetics. The only difference was a larger number of channels in patches from oocytes injected with *sgk*. Another line of evidence that argues against

sgk affecting the P_o was the finding that $\alpha\beta$ channels responded to *sgk* with a 3-fold increase in whole-cell currents. Such an increment could only be accounted by the presence of more functional channels at the cell surface, because the P_o of $\alpha\beta$ channels is already ~1.

Previous studies performed in the rat cortical collecting tubule (20) and in the cell line A6 (21) have shown a great variability in the P_o of ENaC from 0.01 to 0.9 with a mean of 0.5. We consistently found a much higher P_o (0.83–0.87) in oocytes. We do not think that the discrepancy is due to selection for channels with high P_o. After formation of the seal we kept patches for at least 5 min, a period long enough to detect activity even if channels had a very low P_o. If any openings were detected, data from that patch were included in the calculations. Most likely, channels exhibit high P_o because the process or factor that down-regulates ENaC activity in native tissues does not operate in oocytes.

The lack of effect on P_o suggested that *sgk* mainly increases number of channels at the cell surface. However, we also considered an alternative explanation, *i.e.* that *sgk* activates silent channels already present in the plasma membrane. Firsov *et al.* (14) have reported that a large number of channels in the plasma membrane of oocytes are indeed inactive. However, labeling of surface channels with anti-FLAG antibodies and radioactive protein G gave more counts in the oocytes expressing *sgk*. The simplest interpretation of these results is that oocytes injected with *sgk* express more channels on the surface.

Lastly, our results suggest that ENaC is not the direct substrate for *sgk* phosphorylation. We have previously reported that β and γ but not α are phosphorylated and that the phosphoresidues are located in the carboxyl termini of the subunits (12). Here, we showed that the effect of *sgk* remained after deletion of the carboxyl termini from the three subunits. Moreover, channels lacking β or γ responded to *sgk,* indicating that these two subunits do not mediate the effects of *sgk*. Therefore, the only functional significant phosphorylation may take place in the amino terminus of α. We could not completely disprove this possibility because channels with deletions of the first 77 amino-terminal residues or with mutations in five conserved Ser/Thr were not functional. Because phosphorylation studies with ^{32}P are not feasible in *Xenopus* oocytes, a definitive answer awaits experiments in a cell line.

Taken together, the data suggest that *sgk* phosphorylates a yet unidentified protein that in turn mediates translocation of ENaC from an intracellular compartment to the plasma membrane and thus increases sodium permeability.

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