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Regulation of gastric acid secretion by the serum and glucocorticoid inducible kinase isoform SGK3

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Abstract

Background—The serum and glucocorticoid inducible kinase isoform SGK3 is ubiquitously expressed and has been shown to participate in the regulation of cell survival and transport. Similar to SGK1 and protein kinase B (PKB/Akt) isoforms, SGK3 may phosphorylate glycogen synthase kinase GSK3 α,β , which has recently been shown to participate in the regulation of basal gastric acid secretion. The present study thus explored the role of SGK3 in the regulation of gastric acid secretion.

Methods—Experiments were performed in isolated glands from gene targeted mice lacking functional Sgk3 (*sgk3*^{−/−}) or from their wild type littermates (*sgk3*^{+/+}). Utilizing BCECF-fluorescence, gastric acid secretion was determined from Na⁺-independent pH-recovery (pH/min) following an ammonium pulse, which reflects H⁺/K⁺-ATPase activity.

Results—Cytosolic pH in isolated gastric glands was similar in *sgk3*^{−/−} and *sgk3*^{+/+} mice. pH/min was, however, significantly larger in *sgk3*^{−/−} than in *sgk3*^{+/+} mice. In both genotypes, pH/min was virtually abolished in the presence of H⁺/K⁺ ATPase inhibitors omeprazole (100 μ M) or SCH28080 (500 nM). Increase of extracellular K⁺ concentration to 35 mM (replacing Na⁺) or treatment with 5 μ M forskolin increased pH/min in *sgk3*^{+/+} mice to a larger extent than in *sgk3*^{−/−} mice and abrogated the differences between genotypes. Protein kinase A inhibitor H89 (150 nM) decreased pH/min to similarly low values in both genotypes.

Conclusions—Basal gastric acid secretion is significantly enhanced in *sgk3*^{−/−} mice as compared to *sgk3*^{+/+} mice. Thus, SGK3 apparently participates in the regulation of gastric acid secretion.

Keywords

PI3 kinase; GSK3; stomach; H⁺/K⁺ ATPase; K⁺ recycling; H⁺ secretion

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Introduction

Gastric acid secretion is accomplished by the H^+/K^+ ATPase, which pumps H^+ into the gastric lumen in exchange of K^+ [1–4]. The K^+ taken up recirculates into the lumen through apical KCNQ1/KCNE K^+ channels [5–7]. Gastric acid secretion is abolished by genetic knockout of KCNQ1, a finding highlighting the significance of those channels for the maintenance of H^+/K^+ ATPase activity [8].

The gastric KCNQ1/KCNE channels are activated by cAMP [9–12], which thus stimulates gastric acid secretion [2, 4, 13]. Formation of cAMP in gastric glands is inhibited by the phosphoinositide-3-kinase (PI3K) [14]. Accordingly, pharmacological inhibition of PI3K substantially increases gastric acid secretion [14].

The signaling cascade triggered by PI3K involves activation of the phosphoinositide-dependent kinase PDK1 with subsequent activation of the protein kinase B (PKB/Akt) and serum and glucocorticoid inducible kinase (SGK) isoforms [15–21]. Accordingly, gastric acid secretion was enhanced in PDK1 hypomorphic mice [22], i.e. mice expressing some 20% of normal PDK1 [23] and in gene targeted mice lacking functional Akt2 [24]. Moreover, gastric acid secretion is enhanced in animals expressing PKB/Akt/SGK insensitive glycogen synthase kinase 3 [25].

SGK3 has previously been shown to activate several channels and transporters [26–28] including KCNQ1 channels [29]. The phenotype of gene targeted mice lacking functional Sgk3 (*sgk3^{-/-}*) includes deranged postnatal hair follicle development [30–35], decreased intestinal transport [36], slightly delayed growth [34] and decreased locomotion [37]. The present study explored, whether SGK3 participates in the regulation of gastric acid secretion and has been performed in gastric glands from *sgk3^{-/-}* mice and their wild type littermates (*sgk3^{+/+}*).

Materials and Methods

Animals

Generation and breeding of SGK3 deficient mice were described earlier [34]. Sex and age matched mice with age more than 3 months were used for the experiments. All animal experiments were conducted according to the German law for the care and use of laboratory animals and were approved by local authorities.

Treatments

Mice had free access to a standard mouse diet (C1310, Altromin, Lage, Germany) and tap water. Effects of omeprazole (100 μ M), forskolin (5 μ M) and H89 (150 nM) were determined by adding the substances directly to the perfusing solutions at the indicated final concentrations. The substances were obtained from Sigma (Taufkirchen, Germany). The stock solutions of omeprazole and forskolin were prepared in DMSO, whereas H89 was dissolved in distilled water. Sham experiments were performed by adding DMSO or plain distilled water to the perfusing solution at a final dilution of 0.1 %. Omeprazole was added to the luminal side of the parietal cells while incubating with BCECF.

Gastric H⁺ secretion

For isolation of gastric glands animals were fasted for 16 hours prior to experiments on wire grids with free access to tap water. After sacrificing the animals the stomach was removed and cut longitudinally. After washing with standard HEPES solution the fundic and pyloric regions were discarded and the gastric corpus was sliced into 0.3 cm² sections. The tissues were transferred onto the cooled stage of a dissecting microscope and individual glands were carefully detached from the gastric wall by snapping off the tissue using sharpened microdissection tweezers. Care was taken not to touch the apical part of the glands. The glands were attached to a glass coverslip precoated with Cell-Tak adhesive (BD Biosciences).

The solutions, flow lines and perfusion chamber were maintained at 37°C by a thermostatically controlled heating system. The volume of the perfusion chamber was 600 µl and the flow rate was 4 ml/min for all solutions. For digital imaging of cytosolic pH_i isolated individual glands were incubated in a HEPES-buffered Ringer solution containing 10 µM BCECF-AM (Molecular Probes, Leiden, The Netherlands) for 15 min at 37°C. After loading, the chamber was flushed for 5 min with Ringer solution to remove any deesterified dye sticking to the outside of the glands. The perfusion chamber was mounted on the stage of an inverted microscope (Zeiss Axiovert 135), which was used in the epifluorescence mode with a 40 x oil immersion objective (Zeiss Neoplan, Germany). BCECF was successively excited at 490/10 and 440/10 nm, and the resultant fluorescent signal was monitored at 535/10 nm using an intensified charge-coupled device camera (Proxitronic, Germany) and specialized computer software (Metafluor, USA). Between 8 and 20 parietal cells were outlined and monitored during the course of the measurements. The results from each cell were averaged and taken for final analysis. Intensity ratio (490/440) data were converted into pH values using the high-K⁺/nigericin calibration technique [38]. To this end the glands were perfused at the end of each experiment for 5 minutes with standard high-K⁺/nigericin (10µg/ml) solution (pH 7.0). The intensity ratio data thus obtained were converted into pH values using the $r_{\max}$, $r_{\min}$, pK_a values previously generated from calibration experiments performed in isolated gastric glands to generate a standard nonlinear curve (pH range 5 to 8.5).

For acid loading, cells were transiently exposed to a solution containing 20 mM NH₄Cl leading to marked initial alkalinization of cytosolic pH (pH_i) due to entry of NH₃ and binding of H⁺ to form NH₄⁺ [39]. The acidification of cytosolic pH upon removal of ammonia allowed calculating the mean intrinsic buffering power of the cells [39]. Assuming that NH₄⁺ and NH₃ are in equilibrium in cytosolic and extracellular fluid and that ammonia leaves the cells as NH₃:

$$\beta = \Delta[\text{NH}_4^+]_i / \Delta\text{pH}_i$$

where pH_i is the decrease of cytosolic pH (pH_i) following ammonia removal and [NH₄⁺]_i is the decrease of cytosolic NH₄⁺ concentration, which is identical to the concentration of [NH₄⁺]_i immediately before the removal of ammonia. The pK for NH₄⁺/NH₃ is 8.9 [40] and at an extracellular pH (pH₀) of 7.4 the NH₄⁺ concentration in extracellular fluid ([NH₄⁺]₀) is

$19.37 [20/(1+10^{pH_0-pK})]$. The intracellular NH_4^+ concentration ($[NH_4]_i$) was calculated from: $[NH_4]_i = 19.37 \cdot 10^{pH_0-pH_i}$

The calculation of the buffer capacity required that NH_4^+ exits completely. After the initial decline, pH_i indeed showed little further change in the absence of Na^+ and presence of omeprazole, indicating that there was no relevant further exit of NH_4^+ . The pH recovery following the NH_4^+ pulse was determined in the absence of Na^+ to prevent realkalinization by the Na^+/H^+ exchanger.

To calculate the pH/min during realkalinization, a manual linear fit was placed over a narrow pH range (pH 6.7 to 6.9) which could be applied to all measured cells.

The solutions were composed of (in mM): standard HEPES: 115 NaCl, 5 KCl, 1 CaCl₂, 1.2 MgSO₄, 2 NaH₂PO₄ 10 glucose, 32.2 HEPES; sodium free HEPES: 132.8 NMDG⁺Cl⁻, 3 KCl, 1 CaCl₂, 1.2 MgSO₄, 2 KH₂PO₄, 32.2 HEPES, 10 mannitol, 10 glucose (for sodium free ammonium chloride 10 mM NMDG⁺Cl⁻ and mannitol were replaced with 20 mM NH₄Cl); high K⁺ for calibration 105 KCl, 1 CaCl₂, 1.2 MgSO₄, 32.2 HEPES, 10 mannitol, 5 μM nigericin. The pH of the solutions was titrated to 7.4 or 7.0 with HCl/NaOH, HCl/NMDG and HCl/KOH, respectively, at 37°C. Where indicated the K⁺ concentration was increased at the expense of Na⁺/NMDG to 35 mM.

Determination of pH and acid concentration of the gastric luminal contents

The pH of the luminal contents of stomach depends on the amount of acid secreted. Activation of gastric gland leads to decrease in luminal pH and conversely inhibition leads to increase in pH. The luminal pH of the overnight fasted mice therefore serves as a useful indicator of basal gastric acid secretion. To determine the basal intraluminal pH, a previously described method was slightly modified and used [24, 41–43]. Briefly, the mice were fasted overnight on wire grids with free access to tap water and sacrificed under slight ether anaesthesia. The whole stomach was then carefully removed and cut open and the luminal contents were collected by washing the stomach with 10 ml unbuffered isotonic sodium chloride solution (containing additionally with 5 mM KCl and 10 mM glucose). The washings were then centrifuged at 150 g for 5 minutes to remove the particulate matter and the pH of the supernatant solution was measured by a standard pH meter (Knick, Germany). The acid concentration in the solution was measured by titrating the above obtained supernatant solution with 0.01 M NaOH to an endpoint pH 7.0 by monitoring the pH with pH meter and additionally adding a universal pH indicator (Sigma, Taufkirchen, Germany) to the solution. The acid concentration thus obtained was then corrected to the acid concentration of the blank isotonic sodium chloride solution and used for further statistical analysis. The K⁺ ion concentrations in the luminal aspirates were measured by flame photometry (model AFM 5051; Eppendorf, Hamburg, Germany).

Determination of serum gastrin

To determine the serum gastrin levels, the animals were fasted overnight for 16 hours with free access to tap water. About 150 μl of blood specimens were withdrawn into heparinized hematocrit capillaries by retro-orbital puncture during light anesthesia with diethylether

(Roth, Karlsruhe, Germany). Total serum gastrin concentrations were determined by RIA kit, (DiaSorin, Dietzenbach, Germany) according to the manufacturer's instructions.

Histology of gastric tissue

Anesthetized mice were euthanized by cervical dislocation and the skin was cleansed with 70% ethanol. The pars fundica of the mouse stomach was dissected and stomach contents were rinsed out. The tissue was fixed in 4% paraformaldehyde overnight, dehydrated and embedded in paraffin. Four μm thick tissue sections taken from the same region of the pars fundica were prepared, deparaffinized and stained with hematoxylin and eosin. Parietal cell numbers of 10 different areas (each 0.052 mm^2) on different slices per stomach of *sgk3^{+/+}* and *sgk3^{-/-}* mice were determined using a Zeiss Axiovert 200 microscope with a LD Achroplan 20x lens in bright field mode.

Immunofluorescence

For immunofluorescence of KCNQ1 protein abundance in the cell membrane of gastric glands the overnight fasted mice were sacrificed by cervical dislocation under ether anesthesia, and the corpus regions of the stomach were isolated and fixed in 4% paraformaldehyde/0.1 M sodium phosphate buffer (pH 7.4) for two hours and then stored in PBS. After immersion in 30% sucrose for 24 h, 15 μm cryostat sections were taken. After a brief rinse, the sections were incubated for 1 h in normal goat serum with 0.3% Triton X-100. The following washes and incubations were performed in PBS containing 0.3% Triton X-100 and 1% dimethyl sulfoxide. Without rinsing, primary polyclonal goat anti-KCNQ1 antibody (C20, Santa Cruz Biotechnologies), diluted 1:100, was applied to the sections for 12 h at 4°C. After three washes at 10 min, the sections were incubated with donkey anti-goat Alexa488 secondary antibody (Invitrogen/Molecular Probes) for 1.5 h. The slides were analyzed on a Zeiss LSM 510 confocal microscope equipped with a 40x oil immersion lens (NA 1.3).

Flow cytometric analysis (FACS) of KCNQ1 expression in the isolated parietal cells

To isolate the parietal cells from gastric mucosa, a previously described protocol [44] was used. Briefly, the mice were overnight-fasted prior to experiments on wire grids with free access to tap water. The mice were then sacrificed, and the stomach was cut open longitudinally. The fundic and the pyloric regions were quickly discarded and the gastric corpus was taken onto ice-cold standard HEPES buffer. The entire isolation procedure was carried out on ice (4°C). The tissue was then cleaned with standard HEPES buffer and mucus removed. After taking the tissue into fresh standard HEPES buffer, the muscular layer was then separated from the mucosal layer by scraping with a glass slide. The scrapings were then cut into very small pieces with a stainless steel razor and the isolated cells were separated from the tissue debris by gradient centrifugation. The obtained cell pellet was resuspended again in standard HEPES buffer, and the suspension was passed through a stainless steel needle (35 gauge). The cells thus obtained were washed once with standard HEPES buffer and then incubated at 37°C for 30 min first with primary polyclonal anti-goat KCNQ1 antibody, 1:100 dilution (C20, Santa Cruz Biotechnologies) and then with anti-goat FITC secondary antibody, 1:1000 dilution (Santa Cruz Biotechnologies) in an atmosphere with 5% CO_2 . The cells were washed twice and resuspended in Ringer solution containing 5

mM CaCl₂ for flow cytometric analysis (FACS-Calibur from Becton Dickinson; Heidelberg, Germany). Cells were analysed by forward scatter, and FITC-fluorescence intensity was measured in fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Statistics

Intracellular pH, pH recovery and buffer capacity of all parietal cells from one gland were averaged, and the respective mean values were used for further statistical analysis. Data are provided as arithmetic means $\pm$ SEM, *n* represents the number of mice used. All data were tested for significance using Student's *t*-test with Welch's correction or ANOVA (Dunnets test), and only results with *p* < 0.05 were considered statistically significant.

Results

BCECF fluorescence was employed to determine the cytosolic pH in isolated gastric glands from gene targeted mice lacking functional SGK3 (*sgk3*^{-/-}) and from their wild type littermates (*sgk3*^{+/+}). The cytosolic pH was not significantly different between the genotypes under basal conditions and the various experimental conditions (Table 1). The buffer capacity was again similar in gastric gland cells from *sgk3*^{-/-} and *sgk3*^{+/+} mice and was also not significantly altered by the experimental conditions (Table 1).

The H⁺/K⁺ ATPase activity was estimated from the Na⁺-independent pH recovery following an ammonium pulse (pH/min). pH/min was significantly (~ 3-fold) faster in gastric glands from *sgk3*^{-/-} mice than in gastric glands from *sgk3*^{+/+} mice. In both genotypes, pH/min was virtually abolished in the presence of the H⁺/K⁺ ATPase inhibitor omeprazole (100 μ M) (Fig. 1). Treatment with a more specific inhibitor of H⁺/K⁺ ATPase activity, SCH28080 (500 nM) also abolished the proton secretion in both the genotypes. The Na⁺ independent pH recovery values of gastric glands from *sgk3*^{-/-} (0.014 ± 0.007 pH units/minute) and *sgk3*^{+/+} (0.016 ± 0.011 pH units/minute) mice were similar after SCH28080 treatment and thus the difference between the genotypes was abrogated (Table 1).

Enhanced gastric acid secretion in *sgk3*^{-/-} mice is expected to modify the pH and acid content within the stomach. The pH of the luminal contents was significantly lower in *sgk3*^{-/-} mice than in *sgk3*^{+/+} mice. The acid equivalents were again significantly (~ 2-fold) higher in the contents from *sgk3*^{-/-} mice (Fig 2).

In theory, enhanced gastric acid secretion in *sgk3*^{-/-} mice could have resulted from increased release of gastrin. However, the fasting plasma gastrin levels were significantly lower in *sgk3*^{-/-} mice (17.2 ± 1.0 pM, *n* = 7) than in *sgk3*^{+/+} mice (22.6 ± 2.1 pM, *n* = 7), a difference presumably secondary to enhanced gastric acid secretion with subsequent inhibition of gastrin release.

Histology of gastric tissue was obtained to explore whether the enhanced basal gastric acid secretion in *sgk3*^{-/-} mice was secondary to hypertrophy of the glands. The empty stomach weight was not significantly different between *sgk3*^{-/-} (0.25 ± 0.01 gm, *n* = 6) and

sgk3^{+/+} (0.24 ± 0.02 gm, $n = 6$) mice. Histological analysis of the gastric tissue also did not show any difference in the distribution of parietal cells between the two genotypes (Fig 3).

A further series of experiments was performed to explore the role of adenylate cyclase/ cAMP/protein kinase A dependent signaling in the differences of gastric acid secretion between *sgk3^{+/+}* and *sgk3^{-/-}* mice. As illustrated in Fig. 4, treatment of gastric glands with 5 μ M forskolin significantly increased pH/min in gastric glands from *sgk3^{+/+}* mice but had no significant effect on pH/min in gastric glands from *sgk3^{-/-}* mice. Accordingly, the difference between the genotypes was abolished in the presence of forskolin, suggesting a role of adenylate cyclase in the stimulation of gastric acid secretion by SGK3 deficiency.

Conversely, treatment of the gastric glands with the protein kinase A inhibitor H89 (150 nM) significantly decreased pH/min in *sgk3^{-/-}* mice and was without significant effect in *sgk3^{+/+}* mice (Fig. 5). Accordingly, H89 dissipated the difference of pH/min between the two genotypes, pointing to a role of protein kinase A dependent signaling in the stimulation of gastric acid secretion by SGK3 deficiency.

A further series of experiments was performed to elucidate the significance of luminal K⁺ concentration for gastric acid secretion. An increase of the bath K⁺ concentration to 35 mM (replacing Na⁺/NMDG) increased pH/min to similarly high values in gastric glands from *sgk3^{-/-}* and *sgk3^{+/+}* mice (Fig. 6). In the presence of enhanced luminal K⁺, the pH recovery tended to be slightly higher in *sgk3^{-/-}* mice than in *sgk3^{+/+}* mice, a difference, however, not reaching statistical significance (Fig. 6). Accordingly, an increase of extracellular K⁺ concentration almost abolished the difference of gastric acid secretion between *sgk3^{+/+}* and *sgk3^{-/-}* mice.

The increased gastric acid secretion in *sgk3^{-/-}* mice could have resulted from an increase in the luminal K⁺ concentration. To explore that possibility, luminal aspirates were analyzed by flame photometry. The K⁺ ion concentration in the luminal aspirates was indeed significantly higher in *sgk3^{-/-}* mice than in *sgk3^{+/+}* mice (Fig 7a). The increased luminal K⁺ concentration in *sgk3^{-/-}* mice could be due to enhanced activity of apical KCNQ1 channels. The membrane expression of KCNQ1 was thus quantified in isolated parietal cells using flow cytometry. As illustrated in Fig. 7b, c, the membrane abundance of KCNQ1 was indeed significantly higher in parietal cells from *sgk3^{-/-}* mice as compared to *sgk3^{+/+}* mice. Increased membrane expression of the KCNQ1 channel was further confirmed by the immunofluorescence analysis of parietal cells. As shown in Fig. 7d, the cell membrane KCNQ1 abundance was more pronounced in *sgk3^{-/-}* mice than in *sgk3^{+/+}* mice.

Discussion

According to the present observations, the serum and glucocorticoid inducible protein kinase isoform SGK3 contributes significantly to the regulation of gastric acid secretion. SGK3 deficiency is paralleled by enhanced gastric acid secretion and thus, SGK3 exerts an inhibitory effect on gastric acid secretion. Previously, basal gastric acid secretion has been shown to be inhibited by phosphatidylinositol 3 kinase PI3K [14] and phosphoinositide dependent kinase PDK1 [22]. PI3K/PDK1 signaling results in the activation of the serum

and glucocorticoid inducible kinase (SGK) and protein kinase B (PKB/Akt) isoforms [15–21, 27, 45]. Thus, activation of SGK3 presumably contributes to the inhibitory effect of PI3K and PDK1 on basal gastric acid secretion. In a most recent study [24] PKB/Akt2 has similarly been shown to participate in the regulation of gastric acid secretion. Presumably PI3K and PDK1 are effective through parallel activation of SGK3 and Akt2. Along those lines parallel activation of SGK3 and PKB/Akt2 may be similarly important during regulation of hair growth [33].

SGK3 and PKB/Akt2 are known to phosphorylate and thus inactivate glycogen synthase kinase GSK3 [46]. Gastric acid secretion is enhanced in animals expressing PKB/Akt/SGK insensitive glycogen synthase kinase 3 [25]. In those animals, the protein abundance of phosphorylated but not of total protein kinase A was significantly larger than in wild type littermates, indicating that GSK3 stimulates protein kinase A [25]. The effect of SGK3 on gastric acid may thus be due to inhibition of GSK3 with subsequent down regulation of protein kinase A. Accordingly, the difference in gastric acid secretion between *sgk3*^{-/-} mice and *sgk3*^{+/+} mice is reversed by activation of adenylate cyclase with forskolin, which leads to similarly high rates of gastric acid secretion in *sgk3*^{-/-} mice and *sgk3*^{+/+} mice. Conversely, inhibition of protein kinase A by H89 markedly decreases gastric acid secretion to similarly low levels in *sgk3*^{-/-} mice and *sgk3*^{+/+} mice.

In contrast to SGK3 and PKB/Akt2, the SGK isoform SGK1 does not appreciably participate in the regulation of basal gastric acid secretion. Accordingly, basal gastric acid secretion is similar in SGK1 knockout mice and their wild type littermates [47]. SGK1, however, contributes to or even accounts for the stimulation of gastric acid secretion by the glucocorticoid dexamethasone [47]. Stimulation of gastric acid secretion by dexamethasone was blunted by inhibition of PI3K [48], indicating that, similar to SGK3 and PKB/Akt2, SGK1 required stimulation by PI3K. Apparently PI3K plays a dual role in the regulation of gastric acid secretion. At the one hand, PI3K down regulates basal gastric acid secretion by stimulation of SGK3 and PKB/Akt2. On the other hand, PI3K stimulates gastric acid secretion by activating SGK1. In the absence of glucocorticoids, the expression levels of SGK1 are low and the inhibitory effect of PI3K prevails. In the presence of glucocorticoids, SGK1 expression levels are high and PI3K activation leads to increase of gastric acid secretion. Glucocorticoids further modify gastric acid secretion by down regulation of cyclooxygenase [49, 50], an effect similarly enhancing gastric acid secretion [49–52].

The stimulation of gastric acid secretion by SGK3 presumably results from enhanced availability of luminal K⁺ for the H⁺/K⁺ ATPase. The luminal K⁺ concentration is higher in *sgk3*^{-/-} mice than in *sgk3*^{+/+} mice and an increase of extracellular K⁺ concentration enhances gastric acid secretion in *sgk3*^{+/+} mice to similarly high levels as in *sgk3*^{-/-} mice. The increased luminal K⁺ concentration comes at least partially from recycling of cellular K⁺ into the lumen, which is accomplished by K⁺ movement through KCNQ1 channels. Accordingly, KCNQ1 channel membrane abundance is significantly higher in *sgk3*^{-/-} mice than in *sgk3*^{+/+} mice. These channels are critically important for maintenance of gastric acid secretion [8, 53, 54]. The KCNQ1 channels are known to be activated by SGK1 [29, 55, 56] and by protein kinase A [9–12].

In conclusion, basal gastric acid secretion is significantly higher in *sgk3*^{-/-} mice than in *sgk3*^{+/+} mice. The present observations thus reveal a novel regulator of gastric acid secretion.

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List of abbreviations used

BCECF	2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester
DMSO	Dimethylsulfoxide
NMDG	N-methyl D-glucamine
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
FSK	forskolin
omp	omeprazole
SGK	serum and glucocorticoid inducible kinase
PDK	phosphoinositide-dependent kinase
PI3K	phosphoinositol 3 kinase

References

- Hofer AM, Machen TE. K-induced alkalinization in all cell types of rabbit gastric glands: a novel K/H exchange mechanism. *J Membr Biol.* 1992; 126:245–56. [PubMed: 1629906]
- Hou W, Schubert ML. Gastric secretion. *Curr Opin Gastroenterol.* 2006; 22:593–8. [PubMed: 17053435]
- Lee HC, Breitbart H, Berman M, Forte JG. Potassium-stimulated ATPase activity and hydrogen transport in gastric microsomal vesicles. *Biochim Biophys Acta.* 1979; 553:107–31. [PubMed: 36910]
- Yao X, Forte JG. Cell biology of acid secretion by the parietal cell. *Annu Rev Physiol.* 2003; 65:103–31. [PubMed: 12500969]
- Dedek K, Waldegger S. Colocalization of KCNQ1/KCNE channel subunits in the mouse gastrointestinal tract. *Pflügers Arch.* 2001; 442:896–902. [PubMed: 11680623]
- Heitzmann D, Grahammer F, von Hahn T, Schmitt-Graff A, Romeo E, Nitschke R, et al. Heteromeric KCNE2/KCNQ1 potassium channels in the luminal membrane of gastric parietal cells. *J Physiol.* 2004; 561:547–57. [PubMed: 15579540]
- Heitzmann D, Koren V, Wagner M, Sterner C, Reichold M, Tegtmeier I, et al. KCNE beta subunits determine pH sensitivity of KCNQ1 potassium channels. *Cell Physiol Biochem.* 2007; 19:21–32. [PubMed: 17310097]
- Vallon V, Grahammer F, Volkl H, Sandu CD, Richter K, Rexhepaj R, et al. KCNQ1-dependent transport in renal and gastrointestinal epithelia. *Proc Natl Acad Sci U S A.* 2005; 102:17864–9. [PubMed: 16314573]

9. Dong MQ, Lau CP, Gao Z, Tseng GN, Li GR. Characterization of recombinant human cardiac KCNQ1/KCNE1 channels (I (Ks)) stably expressed in HEK 293 cells. *J Membr Biol.* 2006; 210:183–92. [PubMed: 16909339]
10. Kurokawa J, Chen L, Kass RS. Requirement of subunit expression for cAMP-mediated regulation of a heart potassium channel. *Proc Natl Acad Sci U S A.* 2003; 100:2122–7. [PubMed: 12566567]
11. Marx SO, Kurokawa J, Reiken S, Motoike H, D'Armiento J, Marks AR, et al. Requirement of a macromolecular signaling complex for beta adrenergic receptor modulation of the KCNQ1-KCNE1 potassium channel. *Science.* 2002; 295:496–9. [PubMed: 11799244]
12. Potet F, Scott JD, Mohammad-Panah R, Escande D, Baro I. AKAP proteins anchor cAMP-dependent protein kinase to KvLQT1/IsK channel complex. *Am J Physiol Heart Circ Physiol.* 2001; 280:H2038–H2045. [PubMed: 11299204]
13. Ammar DA, Zhou R, Forte JG, Yao X. Syntaxin 3 is required for cAMP-induced acid secretion: streptolysin O-permeabilized gastric gland model. *Am J Physiol Gastrointest Liver Physiol.* 2002; 282:G23–G33. [PubMed: 11751154]
14. Mettler SE, Ghayouri S, Christensen GP, Forte JG. Modulatory role of phosphoinositide 3-kinase in gastric acid secretion. *Am J Physiol Gastrointest Liver Physiol.* 2007; 293:G532–G543. [PubMed: 17569740]
15. Alessi DR, Andjelkovic M, Caudwell B, Cron P, Morrice N, Cohen P, et al. Mechanism of activation of protein kinase B by insulin and IGF-1. *EMBO J.* 1996; 15:6541–51. [PubMed: 8978681]
16. Alessi DR, Cohen P. Mechanism of activation and function of protein kinase B. *Curr Opin Genet Dev.* 1998; 8:55–62. [PubMed: 9529606]
17. Divecha N, Banfic H, Irvine RF. The polyphosphoinositide cycle exists in the nuclei of Swiss 3T3 cells under the control of a receptor (for IGF-I) in the plasma membrane, and stimulation of the cycle increases nuclear diacylglycerol and apparently induces translocation of protein kinase C to the nucleus. *EMBO J.* 1991; 10:3207–14. [PubMed: 1655412]
18. Gamper N, Fillon S, Huber SM, Feng Y, Kobayashi T, Cohen P, et al. IGF-1 up-regulates K⁺ channels via PI3-kinase, PDK1 and SGK1. *Pflugers Arch.* 2002; 443:625–34. [PubMed: 11907830]
19. Kobayashi T, Cohen P. Activation of serum- and glucocorticoid-regulated protein kinase by agonists that activate phosphatidylinositol 3-kinase is mediated by 3-phosphoinositide-dependent protein kinase-1 (PDK1) and PDK2. *Biochem J.* 1999; 339:319–28. [PubMed: 10191262]
20. Kotani K, Yonezawa K, Hara K, Ueda H, Kitamura Y, Sakaue H, et al. Involvement of phosphoinositide 3-kinase in insulin- or IGF-1-induced membrane ruffling. *EMBO J.* 1994; 13:2313–21. [PubMed: 8194523]
21. 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.* 1999; 18:3024–33. [PubMed: 10357815]
22. Rotte A, Bhandaru M, Ackermann TF, Boini KM, Lang F. Role of PDK1 in regulation of gastric acid secretion. *Cell Physiol Biochem.* 2008; 22:725–34. [PubMed: 19088454]
23. Lawlor MA, Mora A, Ashby PR, Williams MR, Murray-Tait V, Malone L, et al. Essential role of PDK1 in regulating cell size and development in mice. *EMBO J.* 2002; 21:3728–38. [PubMed: 12110585]
24. Rotte A, Pasham V, Bhandaru M, Eichenmüller M, Yang W, Qadri SM, et al. Regulation of gastric acid secretion by PKB/Akt2. *Cell Physiol Biochem.* 2010; 25:695–704. [PubMed: 20511715]
25. Rotte A, Pasham V, Eichenmüller M, Yang W, Qadri SM, Bhandaru M, et al. Regulation of basal gastric acid secretion by the glycogensynthase kinase GSK3. *J Gastroenterology.* 2010 in press.
26. Bohmer C, Palmada M, Kenngott C, Lindner R, Klaus F, Laufer J, et al. Regulation of the epithelial calcium channel TRPV6 by the serum and glucocorticoid-inducible kinase isoforms SGK1 and SGK3. *FEBS Lett.* 2007; 581:5586–90. [PubMed: 18005662]
27. 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.* 2006; 86:1151–78. [PubMed: 17015487]

28. Strutz-Seeböhm N, Seeböhm G, Mack AF, Wagner HJ, Just L, Skutella T, et al. Regulation of GluR1 abundance in murine hippocampal neurones by serum- and glucocorticoid-inducible kinase 3. *J Physiol.* 2005; 565:381–90. [PubMed: 15774536]
29. Embark HM, Bohmer C, Vallon V, Luft F, Lang F. Regulation of KCNE1-dependent K(+) current by the serum and glucocorticoid-inducible kinase (SGK) isoforms. *Pflugers Arch.* 2003; 445:601–6. [PubMed: 12634932]
30. Alonso L, Okada H, Pasolli HA, Wakeham A, You T, Mak TW, et al. Sgk3 links growth factor signaling to maintenance of progenitor cells in the hair follicle. *J Cell Biol.* 2005; 170:559–70. [PubMed: 16103225]
31. Campagna DR, Custodio AO, Antiochos BB, Cirilan MV, Fleming MD. Mutations in the serum/glucocorticoid regulated kinase 3 (Sgk3) are responsible for the mouse fuzzy (fz) hair phenotype. *J Invest Dermatol.* 2008; 128:730–2. [PubMed: 17914447]
32. Masujin K, Okada T, Tsuji T, Ishii Y, Takano K, Matsuda J, et al. A mutation in the serum and glucocorticoid-inducible kinase-like kinase (Sgkl) gene is associated with defective hair growth in mice. *DNA Res.* 2004; 11:371–9. [PubMed: 15871460]
33. Mauro TM, McCormick JA, Wang J, Boini KM, Ray L, Monks B, et al. Akt2 and SGK3 are both determinants of postnatal hair follicle development. *FASEB J.* 2009; 23:3193–202. [PubMed: 19433625]
34. McCormick JA, Feng Y, Dawson K, Behne MJ, Yu B, Wang J, et al. Targeted disruption of the protein kinase SGK3/CISK impairs postnatal hair follicle development. *Mol Biol Cell.* 2004; 15:4278–88. [PubMed: 15240817]
35. Okada T, Ishii Y, Masujin K, Yasoshima A, Matsuda J, Ogura A, et al. The critical roles of serum/glucocorticoid-regulated kinase 3 (SGK3) in the hair follicle morphogenesis and homeostasis: the allelic difference provides novel insights into hair follicle biology. *Am J Pathol.* 2006; 168:1119–33. [PubMed: 16565488]
36. Sandu C, Rexhepaj R, Grahammer F, McCormick JA, Henke G, Palmada M, et al. Decreased intestinal glucose transport in the sgk3-knockout mouse. *Pflugers Arch.* 2005; 451:437–44. [PubMed: 15971077]
37. Lang UE, Wolfer DP, Grahammer F, Strutz-Seeböhm N, Seeböhm G, Lipp HP, et al. Reduced locomotion in the serum and glucocorticoid inducible kinase 3 knock out mouse. *Behav Brain Res.* 2006; 167:75–86. [PubMed: 16246437]
38. Waisbren SJ, Geibel J, Boron WF, Modlin IM. Luminal perfusion of isolated gastric glands. *Am J Physiol.* 1994; 266:C1013–C1027. [PubMed: 8178950]
39. Roos A, Boron WF. Intracellular pH. *Physiol Rev.* 1981; 61:296–434. [PubMed: 7012859]
40. Boyarsky G, Ganz MB, Sterzel RB, Boron WF. pH regulation in single glomerular mesangial cells. I. Acid extrusion in absence and presence of HCO₃⁻. *Am J Physiol.* 1988; 255:C844–C856. [PubMed: 2849306]
41. Sidani S, Kopic S, Socrates T, Kirchhoff P, Foller M, Murek M, et al. AMP-activated protein kinase: a physiological off switch for murine gastric acid secretion. *Pflugers Arch.* 2009; 459:39–46. [PubMed: 19621238]
42. Sidani SM, Kirchhoff P, Socrates T, Stelter L, Ferreira E, Caputo C, et al. DeltaF508 mutation results in impaired gastric acid secretion. *J Biol Chem.* 2007; 282:6068–74. [PubMed: 17178714]
43. Gawenis LR, Greeb JM, Prasad V, Grisham C, Sanford LP, Doetschman T, et al. Impaired gastric acid secretion in mice with a targeted disruption of the NHE4 Na⁺/H⁺ exchanger. *J Biol Chem.* 2005; 280:12781–9. [PubMed: 15684419]
44. Rotte A, Bhandaru M, Foller M, Biswas R, Mack AF, Friedrich B, et al. APC sensitive gastric acid secretion. *Cell Physiol Biochem.* 2009; 23:133–42. [PubMed: 19255508]
45. Lang F, Cohen P. Regulation and physiological roles of serum- and glucocorticoid-induced protein kinase isoforms. *Sci STKE.* 2001; 2001:re17. [PubMed: 11707620]
46. McManus EJ, Sakamoto K, Armit LJ, Ronaldson L, Shpiro N, Marquez R, et al. Role that phosphorylation of GSK3 plays in insulin and Wnt signalling defined by knockin analysis. *EMBO J.* 2005; 24:1571–83. [PubMed: 15791206]

47. Sandu C, Artunc F, Grahammer F, Rotte A, Boini KM, Friedrich B, et al. Role of the serum and glucocorticoid inducible kinase SGK1 in glucocorticoid stimulation of gastric acid secretion. *Pflugers Arch.* 2007; 455:493–503. [PubMed: 17618452]
48. Lang PA, Schniepp R, Kirchhoff P, Socrates T, Sidani SM, Geibel JP. PI3 kinase dependent stimulation of gastric acid secretion by dexamethasone. *Cell Physiol Biochem.* 2007; 20:527–34. [PubMed: 17762179]
49. Luo JC, Lin HY, Lu CL, Wang LY, Chang FY, Lin HC, et al. Dexamethasone inhibits basic fibroblast growth factor-stimulated gastric epithelial cell proliferation. *Biochem Pharmacol.* 2008; 76:841–9. [PubMed: 18692028]
50. Yokota A, Taniguchi M, Takahira Y, Tanaka A, Takeuchi K. Dexamethasone damages the rat stomach but not small intestine during inhibition of COX-1. *Dig Dis Sci.* 2007; 52:1452–61. [PubMed: 17221297]
51. Wallace JL, Caliendo G, Santagada V, Cirino G, Fiorucci S. Gastrointestinal safety and anti-inflammatory effects of a hydrogen sulfide-releasing diclofenac derivative in the rat. *Gastroenterology.* 2007; 132:261–71. [PubMed: 17241876]
52. Wang GZ, Huang GP, Yin GL, Zhou G, Guo CJ, Xie CG, et al. Aspirin can elicit the recurrence of gastric ulcer induced with acetic acid in rats. *Cell Physiol Biochem.* 2007; 20:205–12. [PubMed: 17595529]
53. Grahammer F, Herling AW, Lang HJ, Schmitt-Graff A, Wittekindt OH, Nitschke R, et al. The cardiac K⁺ channel KCNQ1 is essential for gastric acid secretion. *Gastroenterology.* 2001; 120:1363–71. [PubMed: 11313306]
54. Lee MP, Ravenel JD, Hu RJ, Lustig LR, Tomaselli G, Berger RD, et al. Targeted disruption of the Kvlqt1 gene causes deafness and gastric hyperplasia in mice. *J Clin Invest.* 2000; 106:1447–55. [PubMed: 11120752]
55. Busjahn A, Seeböhm G, Maier G, Toliat MR, Nurnberg P, Aydin A, et al. Association of the serum and glucocorticoid regulated kinase (sgk1) gene with QT interval. *Cell Physiol Biochem.* 2004; 14:135–42. [PubMed: 15107590]
56. Seeböhm G, Strutz-Seeböhm N, Birkin R, Dell G, Bucci C, Spinoso MR, et al. Regulation of endocytic recycling of KCNQ1/KCNE1 potassium channels. *Circ Res.* 2007; 100:686–92. [PubMed: 17293474]

Fig. 1. pH recovery in parietal cells following an ammonium pulse in the presence and absence of omeprazole

Alterations of cytosolic pH (pH) in parietal cells following an ammonium pulse. To load the cells with H⁺, 20 mM NH₄Cl was added and Na⁺ removed (replaced by NMDG, '0' Na⁺) in a first step (see bars below each original tracing), NH₄Cl removed in a second step, Na⁺ added in a third step and nigericin (pH₀ '7') applied in a fourth step to calibrate each individual experiment.

a. Original tracings illustrating alterations of pH in typical experiments in *sgk3*^{+/+} (left panels) and *sgk3*^{-/-} mice (right panels) in the absence (upper panels) and presence (lower panels) of 100 μM omeprazole.

b. Arithmetic means ± SEM of Na⁺-independent alterations of cytosolic pH (pH/min) in parietal cells from *sgk3*^{-/-} mice (closed bars) and *sgk3*^{+/+} mice (open bars) in the absence (n = 11–13 mice each group, left bars) and presence (n = 4–5 mice each group, right bars) of 100 μM omeprazole. * indicates significant difference between *sgk3*^{-/-} mice and *sgk3*^{+/+} mice, # indicates significant difference between absence (control) and presence of omeprazole.

Fig. 2. Intraluminal pH and acid content of stomach from overnight fasted mice

Arithmetic means $\pm$ SEM (n = 7 mice each group) of the pH of the luminal contents (a) and the acid equivalents in the luminal contents of stomach (b) from *sgk3*^{-/-} (closed bars) and *sgk3*^{+/+} mice (open bars) # indicates significant difference with respect to blank isotonic solution, * indicates significant difference between *sgk3*^{-/-} and *sgk3*^{+/+} mice.

Fig. 3. Histology of gastric tissue

a. paraffin sections showing the parietal cell distribution in stomach tissue from *sgk3^{-/-}* (right panels) and *sgk3^{+/+}* (left panels) mice.

b. Arithmetic means $\pm$ SEM (n = 3–4 mice each group and 10 sections per mouse) of the number of parietal cells in gastric tissue from *sgk3^{-/-}* (closed bars) and *sgk3^{+/+}* mice (open bars).

Fig. 4. Effects of forskolin on pH recovery in parietal cells

a. Original tracings illustrating alterations of pH in typical experiments in *sgk3*^{+/+} (left panels) and *sgk3*^{-/-} mice (right panels) in the absence (upper panels) and presence (lower panels) of forskolin (FSK, 5 μ M).

b. Arithmetic means $\pm$ SEM (n = 4–7 mice each group) of Na⁺-independent alterations of cytosolic pH (Δ pH/min) in parietal cells following an ammonium pulse in the absence (left bars) and presence (right bars) of forskolin (FSK, 5 μ M)) in gastric glands from *sgk3*^{-/-} (closed bars) and *sgk3*^{+/+} mice (open bars). * indicates significant difference between *sgk3*^{-/-} and *sgk3*^{+/+} mice, # indicates significant difference between absence (sham) and presence of forskolin.

Fig. 5. Effects of H89 on pH recovery in parietal cells

a. Original tracings illustrating alterations of pH in typical experiments in *sgk3*^{+/+} (left panels) and *sgk3*^{-/-} mice (right panels) in the absence (upper panels) and presence (lower panels) of PKA inhibitor H89 (150 nM).

b. Arithmetic means ± SEM (n = 4–7 mice each group) of Na⁺-independent alterations of cytosolic pH (Δ pH/min) in parietal cells following an ammonium pulse in the absence (left bars) and presence (right bars) of H89 (150 nM) in gastric glands from *sgk3*^{-/-} (closed bars) and *sgk3*^{+/+} mice (open bars). * indicates significant difference between *sgk3*^{-/-} and *sgk3*^{+/+} mice, # indicates significant difference between absence (sham) and presence of H89.

Fig. 6. Influence of enhanced extracellular K⁺ concentration on pH recovery in parietal cells
a. Original tracings illustrating alterations of pH in typical experiments in *sgk3*^{+/+} (left panels) and *sgk3*^{-/-} mice (right panels) at control (3 mM, upper panels) and at enhanced (35 mM, lower panels) extracellular K⁺ concentration.
b. Arithmetic means ± SEM (n = 3–5 mice each group) of Na⁺ independent alterations of cytosolic pH (Δ pH/min) at normal (3 mM, left bars) and enhanced (35 mM, right bars) extracellular K⁺ concentration in parietal cells from *sgk3*^{-/-} (closed bars) and *sgk3*^{+/+} mice (open bars). # indicates significant difference between high or low K⁺ concentration.

Fig. 7. Expression of KCNQ1 in gastric tissue

a. Arithmetic means $\pm$ SEM (n = 7 mice each group) of the luminal K^+ concentration in the stomach from *sgk3*^{-/-} (closed bar) and *sgk3*^{+/+} mice (open bar). * indicates significant difference between *sgk3*^{-/-} and *sgk3*^{+/+} mice.

b. Original histograms of FACS analysis illustrating the expression of KCNQ1 in *sgk3*^{-/-} and *sgk3*^{+/+} mice.

c. Arithmetic means $\pm$ SEM (n = 4 mice each group) of the KCNQ1-dependent fluorescence intensity in parietal cells from *sgk3*^{-/-} (closed bar) and *sgk3*^{+/+} mice (open bar). * indicates significant difference between *sgk3*^{-/-} and *sgk3*^{+/+} mice.

d. Immunofluorescence of KCNQ1 expression in the parietal cells from *sgk3*^{-/-} (right panels) and *sgk3*^{+/+} (left panels) mice. Antibodies are directed against KCNQ1 (green).

Table 1

Gastric acid secretion in *sgk3^{-/-}* and *sgk3^{+/-}* mice under basal and stimulated conditions.

		Rate of pH recovery (delta pH units/minute)	Intracellular pH (units)	Buffer capacity (mM/pH unit)	Number of mice	Number of cells
Control (Untreated)	<i>sgk3^{+/-}</i>	0.037 ± 0.008	7.26 ± 0.04	32.9 ± 5.9	11	108
	<i>sgk3^{-/-}</i>	0.106 ± 0.017 *	7.25 ± 0.03	35.0 ± 3.3	13	133
omp	<i>sgk3^{+/-}</i>	0.011 ± 0.004 [#]	7.23 ± 0.05	32.9 ± 1.9	5	47
	<i>sgk3^{-/-}</i>	0.017 ± 0.006 [#]	7.29 ± 0.08	31.5 ± 7.4	5	48
SCH28080	<i>sgk3^{+/-}</i>	0.016 ± 0.011 [#]	7.17 ± 0.03	37.3 ± 11.1	3	64
	<i>sgk3^{-/-}</i>	0.014 ± 0.007 [#]	7.13 ± 0.03	34.1 ± 17.0	3	57
Sham (for high K ⁺)	<i>sgk3^{+/-}</i>	0.030 ± 0.005	7.21 ± 0.06	38.6 ± 12.0	4	31
	<i>sgk3^{-/-}</i>	0.107 ± 0.014 *	7.25 ± 0.06	31.3 ± 8.3	4	39
high K ⁺	<i>sgk3^{+/-}</i>	0.322 ± 0.021 [§]	7.22 ± 0.02	32.7 ± 6.0	5	52
	<i>sgk3^{-/-}</i>	0.353 ± 0.004 [§]	7.25 ± 0.10	45.1 ± 14.9	3	39
Sham (for FSK)	<i>sgk3^{+/-}</i>	0.040 ± 0.005	7.20 ± 0.04	44.6 ± 9.9	4	44
	<i>sgk3^{-/-}</i>	0.114 ± 0.025 *	7.26 ± 0.05	36.3 ± 3.9	6	42
FSK	<i>sgk3^{+/-}</i>	0.182 ± 0.048 [§]	7.26 ± 0.04	40.0 ± 6.0	5	68
	<i>sgk3^{-/-}</i>	0.179 ± 0.021	7.24 ± 0.03	40.1 ± 5.5	7	93
Sham (for H89)	<i>sgk3^{+/-}</i>	0.032 ± 0.002	7.24 ± 0.09	32.1 ± 8.0	5	56
	<i>sgk3^{-/-}</i>	0.101 ± 0.021 *	7.23 ± 0.06	36.1 ± 7.7	5	50
H89	<i>sgk3^{+/-}</i>	0.034 ± 0.004	7.23 ± 0.05	32.1 ± 6.5	4	54
	<i>sgk3^{-/-}</i>	0.043 ± 0.004 [§]	7.26 ± 0.05	38.7 ± 7.7	7	69

* indicates significant difference to respective wild type mice.

[#] indicates significant difference to respective control (untreated mice).

[§] indicates significant difference to respective sham.